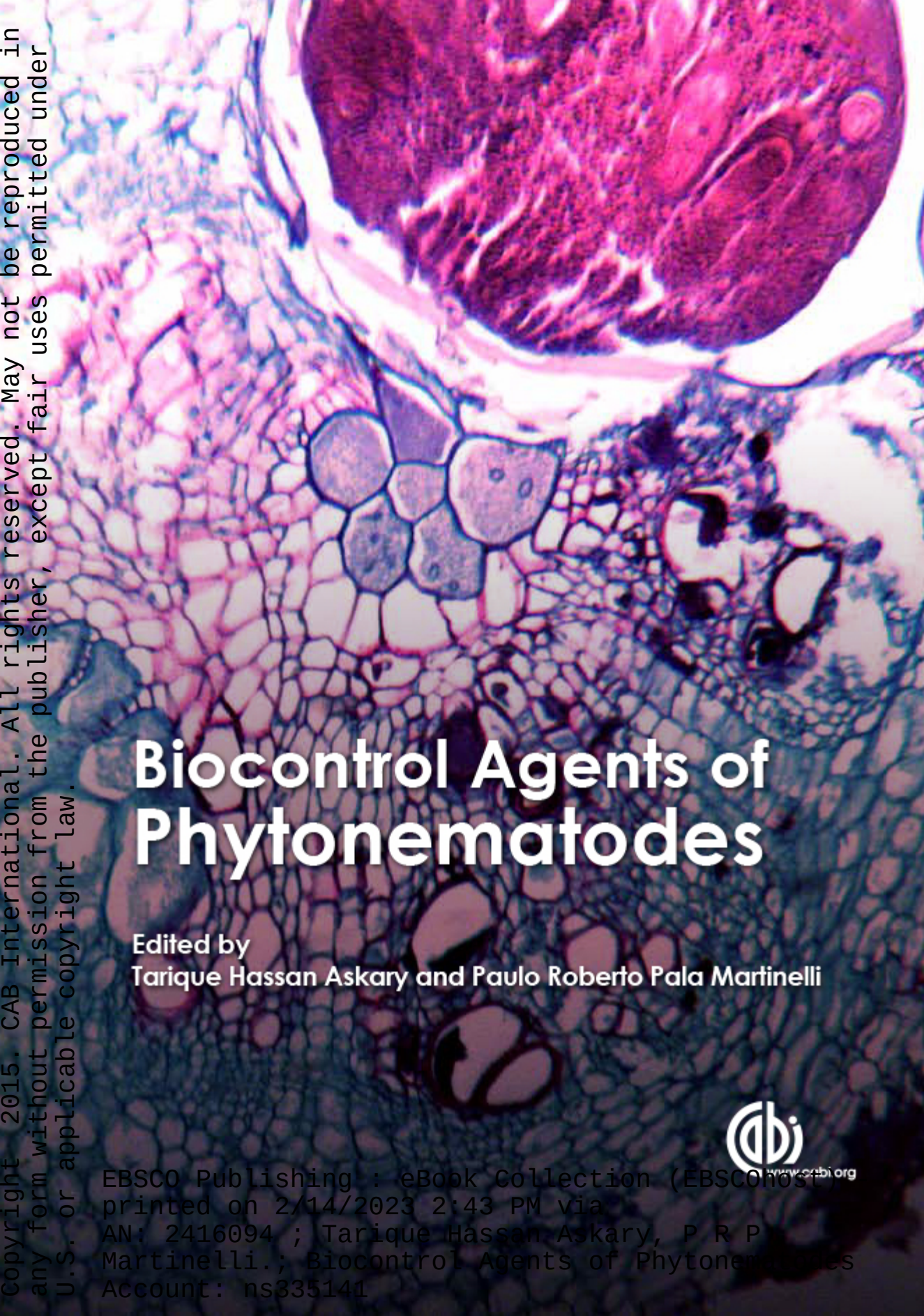

Biocontrol Agents of Phytonematodes

Edited by
Tarique Hassan Askary and Paulo Roberto Pala Martinelli

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Preface

Plant parasitic nematodes pose a serious threat to global crop production. As well as causing significant yield losses, they also impair the quality of crops. Management of these tiny creatures therefore becomes essential. Biological control is a novel, ecofriendly approach, and in the past two decades the use of microbes as biological nematicides has continuously increased. Such beneficial microbes are also called biocontrol agents that potentially target specific nematode hosts.

The role of different microorganisms such as fungi, bacteria, viruses, mites and predatory nematodes as biocontrol agents of phytonematodes is well known and recent years have seen an intensive worldwide search for novel biocontrol agents particularly fungi and bacteria and also advances or breakthroughs in this field, such as the proper formulation of some strains and their commercialization. However, at present there is no book that could provide detailed information about each of them individually, particularly on the two very successful biocontrol agents of phytonematodes, i.e. fungi and bacteria, related to their distribution, virulence, survival biology, formulation, safe application and exploitation. The current volume fulfils these objectives. The idea to compile this book is based on our past two decades of experiences working in the field of biological control. This book has a broader readership but is aimed especially for those undergraduate and postgraduate students and research practitioners having plant nematology as their specialized subject.

In the present volume, 27 experts from ten countries contribute authoritative chapters that capture the full breadth of basic and applied information of some important biocontrol agents that are used or have potential in the management of phytonematodes. The information includes the remarkable developments and latest achievements in this direction. The volume is divided into eight parts comprising of 19 chapters altogether. There are two chapters in Part I. The opening chapter of this book begins by analysing the global impact of phytonematodes on crop production, and in continuation a recent assessment of crop losses caused by these tiny creatures has been estimated based on the previous methodology presented by Prof J.N. Sasser and D.W. Freckman. Chapter 2 illustrates the significance of biocontrol agents with particular emphasis on fungi, bacteria, mites and predatory nematodes in the management of phytonematodes.

In Part II there are six chapters devoted to nematophagous fungi. The first chapter broadly explains the role of nematophagous fungi against phytonematodes. Emphasis of the subsequent chapters shifts to ecology, diversity and geographical distribution, virulence mechanisms, formulation, mass production, application and commercialization of nematophagous fungi. The last chapter of this part deals extensively with regulations and safety measures.

Part III is devoted to nematophagous bacteria and consists of five chapters. The first chapter describes the role of nematophagous bacteria in the management of phytonematodes, and the four additional chapters provide information on the virulence mechanisms, survival biology, field application and commercialization. There is one chapter in Part IV, which outlines the research on nematode-feeding mites. Part V has one chapter that, as well as describing the different modes of action of plant growth-promoting rhizobacteria (PGPR), also reviews the research related to its use in the management of phytonematodes. The only chapter in Part VI provides up-to-date information on arbuscular mycorrhizal fungi (AMF) and highlights the integrated effect of this microorganism in protecting the plants from the deleterious effects of phytonematodes. Part VII has one chapter that covers the biocontrol potential of different groups of predatory nematodes in the control of phytonematodes. Their attributes related to predation and prey-searching capabilities as well as shortcomings in their mass production and application are discussed.

In Part VIII there are two chapters: the first analyses the shortcomings and hindrances coming in the way of commercialization of nematode biocontrol agents, whereas in the final chapter research gaps are identified on the basis of critical issues mentioned in the earlier chapters of this volume, and relevant ideas suggested as to how to utilize biocontrol agents tactically to achieve maximum potential in managing phytonematodes at field level.

We place on record our sincere thanks to all the contributors for their time and effort that helped in making this book possible. We wish to thank those who generously permitted us to use their photographs and also to those who provided necessary information and relevant data needed for several chapters in this book; these persons are acknowledged specifically in the respective chapters. Finally, we express gratitude to Afrin and Monteiro, our long-suffering wives, for their patience and support in innumerable ways during the preparation of this book.

Tarique Hassan Askary and Paulo Roberto Pala Martinelli
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Part I

Phytonematodes and Biocontrol Agents

1 Impact of Phytonematodes on Agriculture Economy

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1.1 Introduction

It is well known that the 2008 global financial crisis, considered by many economists to be the worst financial crisis since the Great Depression of the 1930s, has played a key role in hindering many small and large businesses, and causing a decline in consumer wealth and downturn in economic activity creating high unemployment, unfavourable conditions for new businesses, increase in prices of goods and services and low income per capita. In this context, agriculture, as a far-reaching activity in terms of both economy and sociology throughout world civilization in the history of mankind, has been adversely affected. Combined with applying intensive agriculture systems, usage of arable land for non-agricultural purposes and land degradation through erosion, salination, desertification and pesticides' contamination and/or other by-products of civilization, one will realize that the arable land remaining to feed the growing population will be decreasing on both absolute hectares and hectares per capita (Thomason *et al*., 1983; DEFRA, 2010). In developing countries where hectares per capita is already less than that in developed ones, loss of land for such, or other, reasons will erode their production potential in the agricultural sector. Moreover, individuals and groups of mankind cannot save huge financial resources to continue the policy of securing reasonable development for other reasons widely known all over the world – economic losses due to war damage effected globally, new diseases which demand ample costs to overcome, and non-optimal utilization of available resources. All these in one way or another minimize such resources which could be directed to fill in the gap of agricultural produce. In addition, a continuous challenge is to face an ever-increasing world population with more and better food. Now, experts at almost all levels in developing and more developed countries recognize the seriousness of the world food problem.

Given such a situation, in order to achieve more and better food, further research is required on how to make more ambitious goals or adequately meet ends with less resources or means. From the agricultural economic point of view, ends or objectives may target physical production tools, agricultural services, consumption or profits. Means are concerned with physical resources, funds or organizations that can be used in achieving the objectives. Therefore an economic problem arises only if there are many ends that need satisfying, and when the means

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to achieve these ends are limited. Hence, the central problem in economics is the problem of choice between alternatives. To resolve the problem of choosing between alternatives, economics deals with implementing the alternatives that can best maximize ends, such as physical output, consumer satisfaction and resource allocation and minimize means, like the use of land, labour, capital and organization; i.e. maximization of ends with the given means or minimization of used means for the given ends (Khan, 1972).

In this context, nematodes have rarely been considered or recognized as major limiting factors until all other constraints on yield increase have been removed (Bridge, 1978). Therefore, it is essential that the full spectrum of crop production restrictions is fully and appropriately considered, including the often overlooked phytonematode constraints (Nicol *et al*., 2011) to maximize agriculture output. Some scientists could count more than 1 million kinds of nematodes, rating those next to insects in numbers. About 50% of the known nematodes are marine, 25% live in the soil or freshwater feeding on fungi, bacteria, other saprophytes, small invertebrates or organic matter. About 15% are animal-parasitic nematodes that range from small insects and other invertebrates up to domestic and wild animals and man. Only about 10% of nematodes are parasitic on plants (http://mrec.ifas.ufl.edu/lso/SCOUT/Nematodes.htm). A small percentage of about 3400 known species of plant-parasitic nematodes (Hodda, 2011) is widespread and causes significant losses to crop production (Sasser, 1988; Koenning *et al*., 1999; Nicol *et al*., 2011). Of the remainder, their importance as plant pathogens is unknown; some species have limited distributions and cause localized damage to plants, and some species are recorded only once from their type host and locality. Based on such distributions, their phytosanitary measures seem generally justifiable, depending on whether potential impacts outweigh costs. The latest statistics showed that 250 species from 43 genera satisfied one or more of the criteria to be believed to demonstrate a phytosanitary risk; yet, these species may not cover all species of phytosanitary importance (Singh *et al*., 2013).

1.2 The Nature of Phytonematodes

1.2.1 Habitat, taxonomy, biology, parasitism and injury

Phytonematodes or plant-parasitic nematodes (PPN) live in the soil or in plant tissue, and typically do not move great distances. Anthropogenic movement of the nematodes over great distances may occur via nematode-contaminated materials such as planting material, soil, machinery and organic fertilizers. The majority of PPN affect crops through feeding on or in plant roots, whilst a minority are aerial feeders. Their role is mainly due to direct damage on economically important crop hosts, and ability to act as vectors of viruses or cause complex diseases when in association with other pathogens. The nematodes can damage plants not only through direct feeding and migration within plant tissues, but they also facilitate subsequent infestation by secondary pathogens, such as fungi and bacteria (Powell, 1971). Nematodes can cause significant damage to almost all kinds of crops but due to their subterranean habit and microscopic size they remain invisible to the naked eye (Ngangbam and Devi, 2012). They can occur deep in the soil, usually around the rhizosphere, and many have great capabilities to survive even in the absence of a host (Sasser and Freckman, 1987).

Plant parasitic nematodes of the phylum Nematoda are divided into two orders: Tylenchida and Dorylaimida. All the genera belonging to Dorylaimida (*Xiphinema*, *Longidorus*, *Paralongidorus*, *Trichodorus* and *Paratrichodorus*) are migratory ectoparasites. They are known to attack trees and herbaceous crops, damaging the plants severely by transmitting phytopathogenic nepo- and tobra-viruses. Many economically important genera of migratory endoparasites (*Pratylenchus*, *Radophulus*, *Anguina*, *Aorolaimus*, *Hirschmanniella*, *Hoplolaimus* and *Ditylenchus*) belong to Tylenchida: they damage plants because their movement and feeding inside the roots causes cell death and tissue necrosis. Other phytonematode genera of the same order include *Tylenchorhynchus*, *Helicotylenchus*, *Scutellonema*, *Rotylenchulus*,

Criconemella, *Hemicriconemoides*, *Hemicycliophora*, *Tylenchulus*, *Rhadinaphelenchus* and *Aphelenchoides* (Hunt *et al*., 2005). The most important nematode families of this order, either as models of plant–pathogen interaction or as crop pests, are constituted by sedentary endoparasitic nematodes (Heteroderidae, Nacobbidae). The family Heteroderidae includes the most diffused genera, such as *Globodera*, *Heterodera* and *Meloidogyne*, where the second-stage juvenile (J_2), hatched from an egg, is the 'infective' stage. The J_2 moves through the soil in order to locate new host roots and make entry into them. It penetrates the root tissue, enters into that and establishes a suitable feeding site there. The nematode injects growth-regulating substances into the cells near its head, and consequently some of those cells are enlarged. These 'giant' or 'nurse' cells are the specialized food sources for the nematode. The nematode continuously feeds on giant cells and during this period it starts assuming obesity, becomes immobile or sluggish and advances to maturity (adult stage). *Globodera* and *Heterodera* are cyst-forming nematodes; adult females of cyst-forming nematodes protrude from the roots with the majority of their body, and lay eggs inside the body. Late in the life cycle, the female's external cuticle turns brown (from a whitish to yellowish colour when they are alive) and hardens. These hardened brown dead females are called cysts. The eggs are protected inside the cyst from environmental stresses. The cysts are the means for spreading the infestation. These cysts can be found attached to infested roots or dispersed in the soil.

At the end of their life cycle, females of *Meloidogyne* spp., generally known as root-knot nematodes (RKNs), completely embed themselves into the roots and lay eggs in an external gelatinous matrix, which is fairly visible to the naked eye, outside the roots. Moreover, RKNs can usually induce hypertrophy and hyperplasia of the surrounding tissues, which lead to the formation of galls on roots. Nematodes that are sedentary endoparasitic do not kill parasitized cells and evolve very specialized and complex relationships with their hosts. The feeding sites induced by cyst-forming nematodes are called syncitia, wherein few cells merge as a result of dissolution of their cell walls, whereas *Meloidogyne* spp. induce the formation of few discrete giant cells. Both the feeding sites have the common role, i.e. actively transferring solutes and nutrients toward the developing nematode. However, cyst nematodes do not form root galls. *Meloidogyne* spp. differ from most other sedentary endoparasites by having extensive host ranges, parasitizing more than 2000 plant species, although relatively few host–parasite relationships have been investigated (Molinari, 2009). Other nematodes attack bark and forest trees; the most important representative of this group is the pine wood nematode, *Bursaphelenchus xylophilus*, present in Asia and America and recently in Europe (http://en.wikipedia.org/wiki/Nematode#Taxonomy_and_systematics).

The life cycle of PPN consists of the egg and four juvenile stages, each followed by a moult. Following embryogenesis the first moult occurs within the egg giving rise to a second stage juvenile (J_2). Depending on the availability of suitable temperature and moisture, J_2 emerges out from an egg and moves freely in soil in search of new roots of the same plant or some other plant. A second moult occurs giving rise to J_3. The third moult follows quickly and juveniles develop to J_4. In the case of *Meloidogyne* spp. sex differentiation occurs after the third moult. Females acquire a V-shaped genital primordium, while in males it is I-shaped. The J_3 and J_4 retain the old cuticles as a result of superimposed moult. The pointed tail of J_2 is still visible and hence these are also called the spike-tailed stages. J_3 and J_4 are non-feeding stages as they lack a stylet (Askary, 2008). At the last moult, the adult female becomes sac-like, the stylet reappears and the reproductive system becomes fully developed with the vulval opening making its appearance. Adult males become vermiform, coiled inside the J_4 cuticle, emerge out and migrate out of the root into the soil. They are short lived. The time required for a complete life cycle varies depending on the environmental factors, the host and the nematode species. Usually J_2 is the 'infective' stage, but in the case of *Rotylenchulus reniformis* the immature (pre-adult) female is the infective stage. As an example, this species is a sedentary

semi-endoparasite on roots of many plants. Mature females penetrate partly into roots, leaving the posterior portion of their bodies projecting at the surface (Robinson *et al*., 1997). Juveniles, males and immature females are found in soil. The males are not parasites, they do not feed but they are important for reproduction. *R. reniformis* can survive at least 2 years in the absence of a host in dry soil through anhydrobiosis, a survival mechanism without water (Radewald and Takeshita, 1964). The anterior one-third, more or less, of the young female body penetrates roots and forms a feeding site (syncytium) in the endodermis. The female continues to develop and deposits eggs in a gelatinous matrix outside the roots. Sedentary nematodes tend to be sexually dimorphic, which means the occurrence of individual males and females of the same species is in two different forms. Very common examples are pear, lemon, cyst and kidney-shaped females of *Meloidogyne*, *Tylenchulus*, *Heterodera* and *Rotylenchulus* species, respectively, while their males have a filiform shape. Males are generally smaller than the females. They have a single testis, seminal vesicle and vas deferens that ends at a cloaca or opening. A few male nematodes possess two testes. There are either one or two ovaries in female nematodes, besides receptacles for the male's sperm, a uterus and a vulva. The formation of oocytes, or eggs, takes place in the ovaries. After mating, the sperm is stored in the female's receptacles until needed for fertilization. After fertilization, the female often lays the eggs in the soil. Occasionally, cases of hermaphroditism take place, wherein there is no requirement for a male. The female makes sperm and stores it until she forms eggs (http://www.ehow.com/about_6125285_nematodes-information.html).

Some PPN such as *Aphelenchus* species are parasites of fungi while others, e.g. *Dorylaimus* species, can parasitize algae. However, the most important group of PPN depends on higher plants for their survival and reproduction. The latter group may be divided into parasites of plant shoots (e.g. nematode species of the genera *Aphelenchoides*, *Ditylenchus* and *Anguina*) or roots. Most phytonematode families are obligate root parasites. Nematode families differ in morphology, lifestyles, parasitism and host ranges. According to the degree of penetration of the nematode body inside the root, nematodes are divided into ecto- and endoparasites. Nematodes that insert all or most of the body inside the root are called endoparasites, whilst ectoparasites insert only their stylet inside the root and live and reproduce in soil. If after egg hatching all life stages maintain the ability to move inside or outside the roots, the nematodes are termed migratory; conversely, if a mobile infective stage is followed by developmental immobile stages, nematodes are called sedentary (Molinari, 2009). Galls formed by the sedentary RKNs (*Meloidogyne* spp.) can easily be scratched or cracked, especially on thin or delicate roots of relatively young plants, allowing the entry of soil-borne, disease-causing fungi and bacteria. These galls can be distinguished from the beneficial, 'nitrogen-fixing' nodules on the roots of legumes. Contrary to the root nodules caused by the rhizobia, the nematode galls are true swellings and cannot be rubbed off the roots. The root-lesion (*Pratylenchus* spp.), stunt (*Tylenchorhynchus* spp.) and stubby-root (*Trichodorus* spp. and *Paratrichodorus* spp.) nematodes are aptly named since they cause root lesions, stunted plants and stubby roots, respectively.

All known PPN have a stylet in their mouth to pierce and suck the nutrients from plant cells. Every part of higher plants (roots, stems, trunk, leaves and inflorescences) is susceptible to attack by one or more species. Since most PPN affect root functions, most symptoms associated with them are the result of deficiency in water supply or mineral nutrition to the tops. For example, when plants are severely infected by *Meloidogyne*, the normal root system is reduced to a limited number of severely galled roots with a completely disorganized vascular system. Rootlets are almost completely absent. The roots are seriously hampered in their main functions of uptake and transport of water and nutrients (Netscher and Sikora, 1990). Some PPN produce specific symptoms (Fig. 1.1), but typical ones include reduced growth, wilting, discolouration of leaves and deformation of the roots. The photosynthetic products from shoots to roots are mobilized due to the increase in metabolic activity of giant cells (Hofmann and

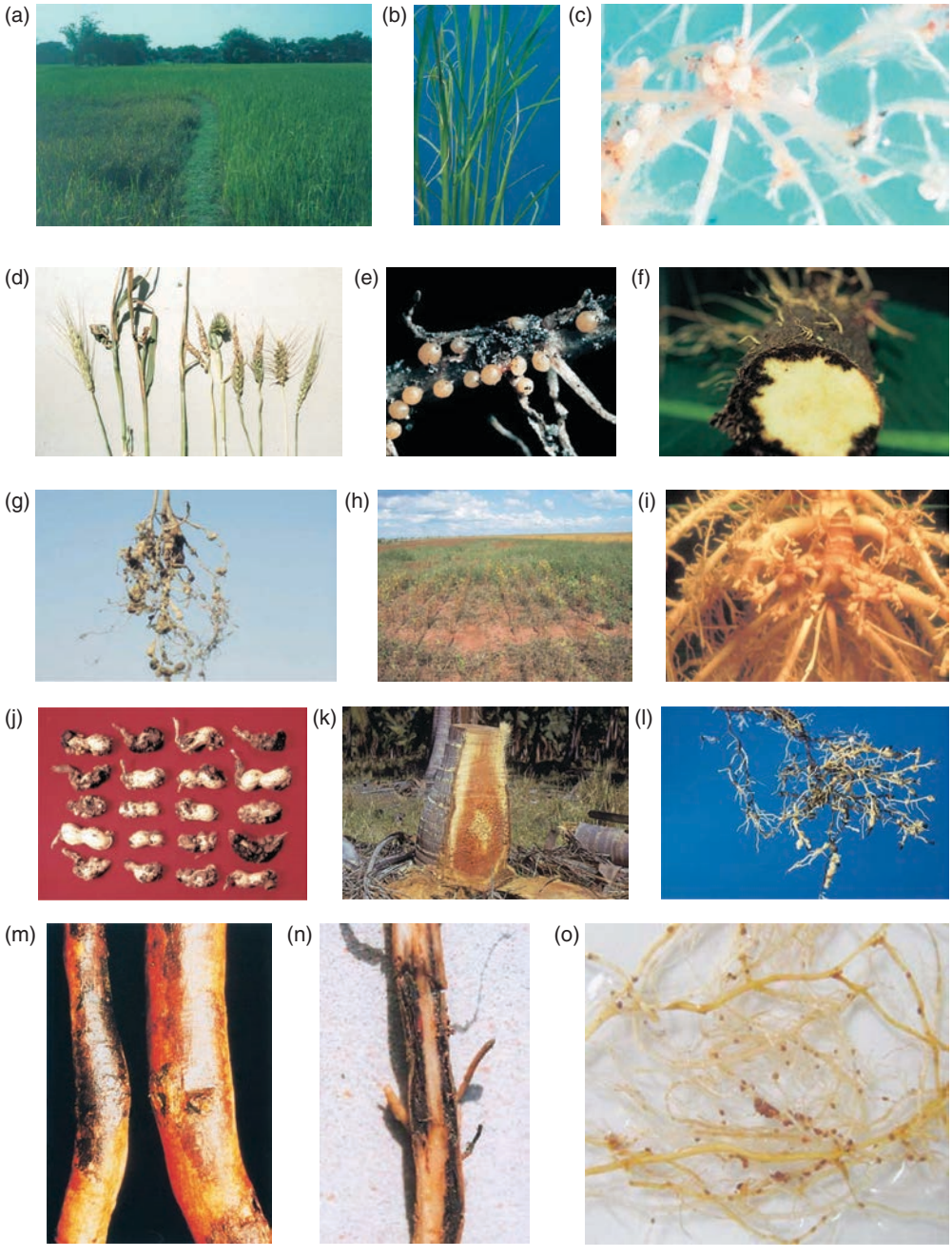

Fig. 1.1. Symptoms of nematode damage. (a) Ufra disease. Brown patch of dead and dying rice caused by *Ditylenchus angustus*. (b) White tip symptoms on rice infested with *Aphelenchoides besseyi*. (c) Symptoms of cereal cyst nematode, *Heterodera avenae*, on wheat roots, showing a bushy appearance. (d) Different stages of *Anguina tritici* infection of wheat in India along with symptoms of 'yellow ear-rot disease' caused by the interaction of the nematode with *Corynebacterium michiganense*. Healthy ears on far right and far left. (e) Cysts of *Globodera rostochiensis* on root of potato. (f) Dry rot disease of yam (*Dioscorea rotundata*) tuber caused by *Pratylenchus coffeae* in Papua New Guinea. (g) Root of a

Grundler, 2007). The yield reduction is manifested in quantity and/or quality changes. Disease complexes, including *Meloidogyne* spp. with other pathogens such as Fusarium wilt, *Rhizoctonia solani* and *Thielaviopsis basicola*, are well-documented (Manzanilla-López and Starr, 2009).

Nematodes can also cause secondary damage. Trees in advanced stages of nematode infection will have little or no new foliage whereas healthy ones show significant flushes, and finally exhibit dieback of progressively larger branches. Gradual decline is typical of nematode-infected turf and pasture. In cases of those plantings which are stunted by nematodes, the weed problems are often worse than in healthy areas, because the crop does not compete with weeds as it should (http://archive.lib.msu.edu/tic/golfd/article/1981feb24.pdf). Admittedly, above-ground damage (chlorosis or yellowing, wilting, stunting, reduced yields, abnormal response to fertilizers, small or sparse foliage, slower recovery from wilting) may look similar to symptoms resulting from plant exposure to other biotic and/or abiotic stresses. Thus, below-ground symptoms are less deceiving but more useful than top symptoms for diagnosis of many nematode problems (http://edis.ifas.ufl.edu/ng006).

1.2.2 Sampling, spatial distribution and contribution in the soil food web

Several factors such as soil moisture and environmental temperature influence the nematode population, its distribution and activities in the soil (Askary *et al.*, 2012). Due to microscopic size and irregular field distribution of nematodes, soil and root tissue samples should be the surest guarantee required to judge whether nematodes are causing poor crop growth or to determine the need for management of root nematodes. Nematode samples may be examined through advisory services to investigate a nematode population in terms of its potential hazard to a future crop or yield loss. The diagnostic service which determines nematode species in a problem situation is rather different from an advisory service. Accuracy of results is only as accurate as the sample. Sampling guidelines are available (http://votivo.us/Nematode-Sampling-Guidelines.pdf). Some difficulties in investigating PPN populations arise from their great complexity and dynamic nature. Each nematode species in a polyspecific nematode community in a given field usually varies in both horizontal and vertical distribution as well as with time (Rickard and Barker, 1982). Yet, management of PPN hinges on detection, the existing species and their population density estimation.

When detection, rather than high population numbers, was the main issue, nematode distribution in three sites followed the Poisson model, while distribution in three other sites followed the negative binomial distribution (Abd-Elgawad and McSorley, 2009); those two distributions are most common for PPN. Their high population levels often follow contagious, negative binomial distribution (Abd-Elgawad, 1992). Yet, the spatial pattern of PPN populations in an agricultural or natural ecosystem has both macro-distributional and micro-distributional components. Macro-distribution within the system is mediated by such factors as the length of time the population has been present in the system, variations in habitat suitability factors such as soil texture, soil moisture and drainage/irrigation patterns, and the selection pressures of differential host plant distributions

Fig. 1.1. Continued.
pigeonpea showing severe galling by *Meloidogyne javanica*. (h) Soybean plants exhibiting chlorosis and early senescence caused by *Heterodera glycines* in North Carolina, USA. (i) 'Stubby-root' symptoms caused by the feeding of *Paratrichodorus minor* on maize. (j) Groundnut pods and a short portion of pegs with light to heavy galling caused by *M. arenaria*. (k) Longitudinal section of old coconut stem showing diffuse reddened tissues caused by *Bursaphelenchus cocophilus* becoming one solid block. (l) *Meloidogyne exigua* galls on coffee roots. (m) Large storage roots of tea displaying necrotic patches caused by *Pratylenchus loosi*. (n) Lesions in banana roots caused by *Radopholus similis*. (o) Brown egg masses of *Rotylenchulus reniformis* on cotton roots (adapted from Luc *et al*., 2005b).

or differential cropping history. The micro-distributional attributes of a nematode population are strongly linked to the life history of the population and feeding strategies (Ferris *et al*., 1990). Sedentary endoparasitic nematodes deposit all their eggs in the same location, frequently in masses, resulting in a highly aggregated spatial pattern. Understanding the spatial distribution patterns of PPN is essential for formulation of efficient sampling plans and for the design and interpretation of field experiments (Ferris, 1978; Noe and Campbell, 1985) including yield loss. Such aggregated distribution of nematodes in soil confounds parametric statistical analysis (Goodell and Ferris, 1980) necessary to study the effects of nematodes on plants. Approaches based on estimates of nematode dispersion indices are being utilized to solve such problems and in the development and evaluation of control measures (Barker and Campbell, 1981). Knowledge of nematode spatial distribution can be used to develop sample size optimization by using equations derived from negative binomial models (McSorley and Parrado, 1982) or by simulation from the database even if the model is not negative binomial (Goodell and Ferris, 1980). These two approaches require specific computer programs and therefore Taylor's power law may be employed to provide a convenient alternative for developing both, i.e. PPN sampling plans and determining transformations of nematode counts (e.g. McSorley *et al*., 1985; Duncan *et al*., 1989; Abd-Elgawad, 1992; Abd-Elgawad and Hasabo, 1995). Yet, Chi-squared test for goodness-of-fit applied on *Heterodera* spp. population data of all tested plots in an Egyptian wheat field proved that the Poisson model was a good fit ($P \leq 0.05$) to the original nematode counts when Taylor's power law was not significant (Abd-Elgawad and Mohamed, 2013). On the other hand, soil nematodes can be a tool for testing ecological assumptions and approaching biological mechanisms in soil because of their pivotal role in the soil food web and linkage to ecological processes. In natural and agricultural soil, the aquatic organisms, i.e. plant as well as free-living nematodes, depend on thin water films to live and move within existing pathways of soil pores of 25–100 µm diameter, which makes them amenable candidates for such tasks. Ecological succession is one of the most tested community ecology concepts, and a variety of nematode community indices have been proposed for environmental monitoring purposes (Neher, 2010). Yet, the functional role of nematodes, determined by their metabolic and behavioural activities, may be categorized as ecosystem services, disservices or effect-neutral (Ferris, 2010). Among the disservices pertained to nematodes are overgrazing, which reduces supplies of prey organisms, and plant-damaging herbivores, which diminish carbon fixation and availability to other organisms in the food web. However, management to enhance potential disservices of specific nematodes results in unintended but long-lasting reduction of the services of others. Hence, beneficial roles of nematodes may be upgraded by environmental stewardship that dictates greater biodiversity and, consequently, complementarity and continuity of their favours. This could be achieved through better approaches of optimal foraging, theories of biogeography, colonization and niche partitioning by nematodes. Ecological assumptions pertained to strategies of coexistence of nematode species sharing the same resource have potential uses for better biocontrol benefits and application of organic matter to attain disease control. Research should be focused on nematodes in natural and agricultural soils to synchronize nutrient release and availability related to plant needs, to test ecological hypotheses, to apply optimal foraging and niche-partitioning strategies for promising biocontrol tactics, to blend organic amendments to control phytonematodes, to monitor environmental and restoration condition, and to obtain better predictive models for decision-makers about optimum land-use (Neher, 2010). PCR-based approaches to identify and quantify species (real time qPCR and next generation sequencing) greatly expand the ability to investigate such food web interactions because there is less need for wide taxonomic expertise within research programmes (Campos-Herrera *et al*., 2013a).

1.3 Phytonematode Genera, Species and Races

1.3.1 Economically important plant parasitic nematodes worldwide

Around 4100 species of PPN have been described (Decraemer and Hunt, 2006) and, collectively, they impose an important restriction on the delivery of global food security. A questionnaire was conducted to explore perspectives on nematology (Sasser and Freckman, 1987) fuelled by a well-managed and funded scheme of the International *Meloidogyne* Project (IMP). The respondents, all nematologists, were asked to rank the five most damaging genera of PPN occurring in their country or state. Based on the number of first, second, third, fourth and fifth place votes, a weighted index was calculated by giving first place votes a score of 5, second place votes a score of 4, third place votes a score of 3, fourth place votes a score of 2 and fifth place votes a score of 1. The total weighted votes each nematode genus received are given in parentheses. On a worldwide basis, the ten most important genera of plant parasitic nematodes were reported to be *Meloidogyne* (1375), *Pratylenchus* (782), *Heterodera* (606), *Ditylenchus* (251), *Globodera* (244), *Tylenchulus* (233), *Xiphinema* (205), *Radopholus* (170), *Rotylenchulus* (142) and *Helicotylenchus* (122). For the countries represented at IMP, the order of importance of the various genera was somewhat different, although *Meloidogyne* was still first and *Pratylenchus* and *Heterodera* were high on the list. In Europe, however, *Heterodera* (161), *Globodera* (156), *Meloidogyne* (100), *Ditylenchus* (93), *Pratylenchus* (88), *Aphelenchoides* (26), *Xiphinema* (26), *Trichodorus* (23), *Longidorus* (17) and *Tylenchulus* (8) were estimated to be most damaging to plants (Sasser, 1988). This order of importance of the various genera is still fairly representative for most regions of the world but it was carried out more than a quarter of a century ago.

A recent comprehensive survey, from around 1100 individual votes, was conducted at the end of 2012. Members of Nematology Societies across the world, and alumni from major postgraduate plant nematology courses, were asked to select their top five phytonematodes. More than 225 responses, representing around 1100 individual votes, were received, from which the top ten PPN species based on scientific and economic significance were obtained. All cyst as well as all root-knot nematode species were grouped in order to avoid repetition. It also allowed some less familiar but still economically significant PPN to be recorded (Jones *et al*., 2013). Admittedly, any such list will not be definitive as economic importance varies from one region to another of the world in which a researcher is located. However, the survey was designed to include researchers from as many parts of the world as possible to avoid this problem. The top ten list emerging from the survey was composed of: (i) RKNs (*Meloidogyne* spp.); (ii) cyst nematodes (*Heterodera* and *Globodera* spp.); (iii) root lesion nematodes (*Pratylenchus* spp.); (iv) burrowing nematode (*Radopholus similis*); (v) stem and bulb nematode (*Ditylenchus dipsaci*); (vi) pine wilt nematode (*Bursaphelenchus xylophilus*); (vii) reniform nematode (*Rotylenchulus reniformis*); (viii) dagger nematode (*Xiphinema index*, the only virus vector nematode to make the list); (ix) false root-knot nematode (*Nacobbus aberrans*); and (x) rice white tip nematode (*Aphelenchoides besseyi*) (Jones *et al*., 2013). However, some important nematodes just missed out on being included in this list. So, honourable mention should go to several nematodes including spiral nematodes, *Helicotylenchus* spp. (Subbotin *et al*., 2011) and the stubby root nematodes, *Trichodorus* spp., the vector of *Tobacco rattle virus* (Decraemer and Geraert, 2006; in Jones *et al*., 2013). Moreover, other important PPN may require specific climatic conditions to thrive and consequently prevail in specific regions. For example, nematode parasites of plant shoots (species of *Aphelenchoides*, *Anguina* and *Ditylenchus*) are generally not favoured by arid and semi-arid regions because high relative humidity is the requirement to make them able to move on plant shoots. In this context, *A. besseyi* was found in Egypt only near the shores of the Mediterranean Sea (Amin, 2001) whereas *D. dipsaci* was detected on lucerne plants at a land depression in Al-Gassium, Saudia Arabia (M.F.M. Eissa, The National Research Centre, Egypt, 2013, personal communication).

The latest statistics (Singh *et al*., 2013) compile data on PPN hosts, geographic distribution,

yield loss and quarantine status from published records. Such statistics consider a PPN species of phytosanitary importance if it can meet at least one of the following criteria: (i) recorded in the peer-reviewed scientific literature as pathogenic (causing disease) or parasitic (infecting or associated as ectoparasites) of an economically important crop host; (ii) recorded in the peer-reviewed scientific literature acting as a vector of, or forming disease complexes with other pathogens such as bacteria, fungi and viruses; and (iii) currently on an official list of regulated pests, for at least one country. Accordingly, the following genera and number of species (in parentheses) were considered as posing phytosanitary risk (Singh *et al*., 2013): *Achlysiella* (1), *Anguina* (8), *Aphasmatylenchus* (1), *Aphelenchoides* (12), *Aphelenchus* (1), *Belonolaimus* (2), *Bitylenchus* (3), *Bursaphelenchus* (4), *Cactodera* (3), *Ditylenchus* (8), *Dolichodorus* (1), *Globodera* (3), *Helicotylenchus* (7), *Hemicriconemoides* (3), *Hemicycliophora* (3), *Heterodera* (25), *Hirschmanniella* (5), *Hoplolaimus* (5), *Ibipora* (3), *Longidorus* (10), *Macroposthonia* (2), *Meloidogyne* (38), *Merlinius* (3), *Nacobbus* (1), *Neodolichodorus* (2), *Paralongidorus* (2), *Paratrichodorus* (11), *Paratylenchus* (3), *Pratylenchus* (24), *Punctodera* (3), *Quinisulcius* (3), *Radopholus* (5), *Rotylenchulus* (3), *Rotylenchus* (1), *Scutellonema* (5), *Sphaeronema* (1), *Subanguina* (3), *Trichodorus* (5), *Tylenchorhynchus* (8), *Tylenchulus* (2), *Vittatidera* (1), *Xiphinema* (15) and *Zygotylenchus* (1).

Certain aspects concerning the PPN species category, intraspecific categories and races should be considered. Significant progress has been made especially for the most common genera. The North Carolina Differential Host Test (Taylor and Sasser, 1978) is designed to identify the most widely distributed *Meloidogyne* species and races. It can be used for surveys of such new populations, and is especially useful for their identification from new locations or new hosts in the territory under survey. Tobacco, cotton, pepper, watermelon, groundnut and tomato are used by this test but additional hosts, such as maize, were added later by others. For the cyst nematodes, special attention is paid to the existence of cryptic or sibling species, the interspecific hybridization possibility, the reduced reproductive compatibility phenomenon of certain populations within a species and the loss of subspecies and race identity in mixtures (Sturhan, 1985). Whereas 'race' is considered as a population concept, pathotypes are known as virulence phenotypes. Races are genetically variable groups of related populations, but pathotypes are *a priori* 'artificial' entities, designated for practical reasons and identified by constancy of their relevant characters. Although the use of resistant potato cultivars represents an effective method to enforce potato cyst nematode (PCN) control, a number of virulent populations have been identified and characterized as specific pathotypes, according to their ability to reproduce on a set of differential potato clones or cultivars carrying different resistance genes. Continued attempts to differentiate *Globodera* isolates by means of restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD-PCR), amplified fragment length polymorphism (AFLP) and isoelectric focusing pattern methods are in progress (e.g. Bendezu and Evans, 2001; Sedlák *et al*., 2004; Molinari *et al*., 2010) to improve the use of such resistant cultivars.

1.3.2 Economic thresholds for damage by plant parasitic nematodes

The term 'nematode economic threshold' is defined as the density of nematode population at which the value of the damage caused is equal to the cost of the control. Hence, at population levels less than equal to the economic threshold, there is no advantage to nematode control because the cost would be greater than/equal to the increased yield (return) of the crop. Yet, this concept should be supported by the abundance of information on the relationship between nematode densities and plant-damage functions, availability of the assay of PPN population densities in a field, and resources devoted to both to arrive at the decision and nematicide application. Damage thresholds (the point at which yield losses make crop production uneconomic) can be as low as 1 egg/100 cm^3 soil (Greco and Di Vito, 2009). A practical example of damage threshold level for species of root-knot (*Meloidogyne*), sting (*Belonolaimus*), lesion (*Pratylenchus*), lance

(*Hoplolaimus*), spiral (*Helicotylenchus* and *Peltamigratus*), stubby root (*Trichodorus* and *Paratrichodorus*), cyst (*Heterodera* and *Globodera*), stunt (*Tylenchorhynchus*), ring (*Mesocriconema*), sheath (*Hemicycliophora*), burrowing (*Radopholus*), citrus (*Tylenchulus*), reniform (*Rotylenchulus*) and awl (*Dolichodorus*) nematodes is given for common field crops, vegetables and fruits (Table 1.1) and for warm-season turf grasses (Table 1.2) in Florida, USA. These tables indicate minimum numbers of plant nematodes per 100 cm^3 of soil at which the crop is at some risk of nematode damage; such pre-plant thresholds may not be useful on established plants. Action thresholds necessitate RKN control if any individual of *Meloidogyne* spp. was found per 100 cm^3 of tomato-planted soil in Florida as in other countries like Egypt (Anonymous, 2012a).

Estimates of threshold levels require various economic counts. Therefore, predicting yield losses and calculating economic thresholds for most nematode/crop problems is not yet possible in many regions. There is a need for more field-based information on the relationship between population densities of nematode and crop performance. Various approaches to obtain such data are described (http://www.fao.org/docrep/v9978e/v9978e07.htm). It is suggested to conduct pot studies to determine basic information on yield-loss relationships and related thresholds, but due to environmental differences and interactions, field trials are also needed to achieve accurate results. The field approach needs a wide range of initial nematode populations, a uniform field, a large number of plots (100 or more), and the plots to be part of an otherwise uniform crop. The need for this type of information is still apparent since re-directions in the type and choice of applicable nematicides are underway due to environmental issues obstructing the use of traditional chemicals. Yet, some of such nematicides banned in developed countries are still used in less developed ones (Abd-Elgawad, 2008).

1.3.3 Examples of damage by plant parasitic nematodes

Striking examples of damage and yield losses caused by PPN are RKN species on leguminous and cucurbit crops. The cereal cyst nematodes (CCN), especially *Heterodera avenae*, are considered a major biotic factor affecting the wheat and barley production systems in most of their areas. Although CCN have been found causing economic damage exclusively in light soils, such damage can also be done irrespective of soil type in a situation where cereal cropping intensity exceeds a certain limit (Nicol, 2002). Also, the presence of high populations of PPN around the rhizosphere of many fruit and forest crop nurseries led to the assumption that these deleterious tiny creatures may be a contributing factor in the declining health of these plants (Askary and Haider, 2010; Askary *et al.*, 2013). Research on the distribution and economic impact demonstrates that several species of PPN particularly RKN and CCN cause important losses especially under rainfed conditions and limited irrigated regions in Australia, western Asia, North Africa, China and the Pacific Northwest of the USA. Yield losses due to CCN are: 15–20% on wheat in Pakistan (Maqbool, 1988), 40–92% on wheat and 17–77% on barley in Saudi Arabia (Ibrahim *et al*., 1999), 20% on barley and 23–50% on wheat in Australia (Meagher, 1972), 24% in the Pacific Northwest of the USA, 26–96% in Tunisia and 42% in several rainfed winter wheat locations in Turkey (Nicol, 2002; Nicol and Rivoal, 2008; Nicol *et al*., 2011). In Iran under microplot field trials yield losses of 48% were found on common winter wheat over two wheat seasons (Hajihasani *et al*., 2010). The damage to the crop becomes severe when PPN interact with a fungal pathogen. Hassan *et al.* (2012) investigated the interactions between *H. avenae* and *Fusarium culmorum* on growth and yield components of durum wheat. The reduction in grain yield due to individual treatment of *H. avenae* and *F. culmorum* was 12.3 and 25.5%, respectively. But there was a 38.4% reduction when *H. avenae* and *F. culmorum* were inoculated simultaneously, which indicates combined effect on yield losses due to the two pathogens. The interaction was synergistic when the nematode inoculation was done 1 month prior to fungus. There was a 43.8% reduction in grain yield that exceeded the sum of individual loss induced by the nematode and fungus alone. However, antagonistic interaction was observed when the fungus was added 1 month prior to nematode inoculation

Table 1.1. Minimum numbers of plant nematodes per 100 cm^3 of soil at which control (resistant variety, rotation, nematicide) is recommended when only one important nematode pest (genus) of that crop is present; lower levels may justify control when two or more pathogenic genera are found together. Values for annual crops are pre-plant densities (adapted from W.T. Crow, University of Florida, Florida, USA, 2013, personal communication).

Crop	Root-knot	Sting	Lesion	Lance	Spiral	Stubby-root	Cyst	Stunt	Ring	Sheath	Burro-wing	Citrus	Reni-form	Awl
Field maize	80	1	150	40	–	10	–	80?	500	?	–	–	–	10
Cotton	80	1	80	150	–	?	–	–	–	–	–	–	80	?
Groundnuts	1	–	1	–	–	–	–	–	150	–	–	–	–	?
Soybeans	1	1	80	?	–	?	1	–	–	–	–	–	1	?
Sugarcane	10	10	40	?	500	40	–	500	500	?	–	–	–	10
Tobacco	1	–	40	–	–	–	–	–	–	–	–	–	–	–
Citrus	–	–	10	–	–	–	–	–	–	–	1	1	–	–
Peach	1	–	10	–	–	–	–	–	80	–	–	–	–	–
Bean	1	1	–	?	–	1	–	–	–	–	–	–	1	10
Carrots	1	1	10	–	–	–	–	–	–	–	–	–	–	10
Celery	1	1	–	–	–	1	–	–	–	–	–	–	–	10
Sweetcorn	10	1	150	40	–	1	–	80?	500	–	–	–	–	10
Crucifers	1	1	80	–	–	1	1	–	–	–	–	–	–	10?
Cucurbits	1	1	–	–	–	–	–	–	–	–	–	–	–	10?
Lettuce, endive, aubergine, okra, onion, pepper, spinach, potato	1	1	80	–	–	1	–	40	–	–	–	–	–	10
Sweet potatoes	1	–	–	–	–	–	–	–	–	–	–	–	–	?
Strawberries	1	1	80	–	–	–	–	–	–	80	–	–	–	?
Tomatoes	1	1	40	–	–	1	–	–	–	80	–	–	1	10

?, damaging level uncertain; –, not believed to cause significant damage at any level.

Table 1.2. Risk thresholds for warm-season turf grasses used by the University of Florida nematode assay laboratory (adapted from W.T. Crow, Florida, USA, 2013, personal communication). These nematode thresholds are based upon numbers per 100 cm^3 of soil extracted using a sugar-flotation with centrifugation method and are based upon nematodes, grasses and conditions in Florida only. They may not apply in other states or regions. While bahia grass is a host for many of these nematodes, it is very tolerant to them and seldom is damaged; therefore, no thresholds are given. Other nematodes such as dagger, lesion, stunt, etc. may damage turf in Florida, but damage from these is very rare so thresholds are not listed.

	Root-knot (*Meloidogyne*)		Sting (*Belonolaimus*)		Lance (*Hoplolaimus*)		Stubby-root (*Paratrichodorus*)		Stubby-root (*Trichodorus*)		Spiral (*Helicotylenchus*)		Spiral (*Peltamigratus*)		Ring (*Mesocriconema*)		Sheath (*Hemicycliophora*)		Sheathoid (*Hemicriconeмoides*)		Awl (*Dolichodorus*)		Cyst (*Heterodera*)	
Turf-grass type	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H	M	H
Bermuda	80	300	10	25	40	120	150	300	40	120	700	1500	150	300	500	1000	150	300	500	1000	10	25	–	–
Ultradwarf Bermudagrass	40	200	5	20	40	120	150	300	40	120	700	1500	150	300	500	1000	150	300	500	1000	5	20	–	–
Zoysia	80	300	10	25	40	120	150	300	40	120	700	1500	150	300	500	1000	150	300	500	1000	10	25	–	–
Seashore paspalum	80	300	10	25	40	120	150	300	40	120	300	700	150	300	500	1000	150	300	500	1000	10	25	–	–
St Augustine	80	300	25	50	40	120	40	120	40	120	700	1500	150	300	500	1000	150	300	500	1000	10	25	10	40
Centipede	80	300	10	25	40	120	150	300	40	120	700	1500	150	300	150	300	150	300	150	300	10	25	–	–

–, not believed to cause significant damage.
M, turf is considered at moderate risk of damage. Damage may become evident if the turf is placed under stress conditions.
H, turf is considered at high risk of damage. Root systems are likely damaged and turf quality may be declining.

and the reduction in grain yield was 33.3%, less than the loss caused by the nematode and fungus alone. The nematode–fungus interaction resulted in decreasing final population densities for the nematode especially when inoculation of fungus was done 1 month prior to nematode. The highest severity of fungus infection (2.8) and disease index (91.7%) was when the nematode inoculation was done 1 month prior to fungus. Nicol and Rivoal (2008) reported 50% yield loss on barley due to *H. latipons* in Cyprus. Minimum relative yields were 0.4 at initial population (P_i) of 40 *Heterodera filipjevi* eggs and J_2/g soil in the first year and 0.45 at the P_i of 64 in the second year for the grain yield of winter wheat in Iran (Hajihassani and Hajihassani, 2010) where 40% of the surveyed fields were infested with at least one species of either *H. filipjevi* or *H. latipons* (Hajihasani *et al*., 2011). Because the cysts of *H. avenae*, *H. latipons* and *H. filipjevi* are fairly similar in morphology, it is possible that damage caused by the recently described nematode species has previously been attributed to *H. avenae* (Mor *et al*., 1992; Nicol *et al*., 2011). In other countries like Egypt, though CCN were found in each of 60 nematode samples, each consisted of three subsamples in a wheat field (Abd-Elgawad and Mohamed, 2013), their yield losses have not been accurately investigated. Staggering annual yield losses of US$9 million in India, £3 million in Europe and AUS$72 million in Australia have been calculated as being caused by *H. avenae* (Van Berkum and Seshadri, 1970; Nicol *et al*., 2011). The losses in Australia are now greatly reduced due to control of the disease with resistant and tolerant wheat cultivars. For soybean, yields suppressed in the USA due to diseases during 1996–2009 were estimated (http://aes.missouri.edu/delta/research/soyloss.stm); the yield loss (million bushels) was around 172, 120 and 118 due to the soybean cyst nematode, *Heterodera glycines*, and 10, 7 and 8 due to RKN and other nematodes in 2008, 2009 and 2010, respectively.

Jones *et al*. (2013) advocated the importance of *M. graminicola* as the main species infecting upland (rainfed) and lowland (irrigated) rice. This particular species has been found well adapted to flooded fields causing a yield loss of up to 87%. The species is difficult to control because of its short life cycle and wide host range, which includes the presence of different weeds that are common in rice fields. Interaction of root-knot/reniform nematode with wilt fungus *Fusarium*, resulting in crop yield loss has been reported by several workers (Jain and Sharma, 1996; Singh *et al*., 2004). In India, Askary and Ali (2012) made an attempt to find out the effects of RKN *M. javanica* alone, sequentially and simultaneously with *F. udum* on wilt incidence and growth characters of five wilt-resistant accessions of pigeonpea. All the accessions were found susceptible to nematode infection. It was observed that the greatest reduction in plant growth parameters was observed when nematode and fungus were applied simultaneously, followed by application of nematode 7 days prior to fungus and fungus 7 days prior to nematode. This led to the conclusion that there is a loss of resistance to wilt in pigeonpea when both RKN and wilt fungus are present in soil. Such findings have also been reported by earlier research workers (Salam and Khan, 1986; Sharma and Nene, 1990).

1.3.4 Plant parasitic nematode distribution and population density: implications and management

The distribution of nematodes within any site is mostly very irregular. In parallel, the shape, size and distribution of areas showing symptoms of nematode diseases should be highly irregular within the field. Movement of a nematode on its own is limited to 1–2 m/year. As for their hosts, different crops may have a broad or narrow geographical plasticity, as do their pests. PPN may vary according to region, continent and climate.

Nematode genera and species show greater diversity in subtropical and tropical countries as compared to temperate ones due to the wide diversity of crops and agricultural systems (e.g. Luc *et al*., 2005a; Askary *et al*., 2011). Generally, PPN have a shorter life cycle, which results in a more rapid population explosion than in temperate areas. In temperate areas, *Heterodera* spp. generally produces one or two generations per year, but in tropical areas such as West Africa, *H. oryzae* produces one

generation every 25 days (Merny, 1966; Luc *et al*., 2005a). For each susceptible crop, there are some main PPNs.

Nematode densities differ among soil types, plant species/cultivars and other environmental factors. Geographical distributions, frequency of occurrence and population density of nematode species are good indicators of potential crop damage and economic impact. Some serious PPN species, such as both the cereal and the potato cyst nematodes, may be endemic to definite areas planted by these crops but not found in others where the same crops are grown. Some crops are produced in regions of varying levels of economy, leading to different levels of nematode management, often as a consequence of awareness as well as the availability of options/funds for their management. Therefore many factors can control nematode frequency of occurrence and their population density resulting in great variations in the distribution of nematode genera/species on a global scale. Some are cosmopolitan, such as certain *Meloidogyne* spp., but others are fairly restricted geographically, e.g. *Nacobbus* spp., or are highly host specific, such as *Heterodera carotae* which attacks carrots. Some crops may have very few nematode pests, e.g. the doum palm and mangrove tree, while others have a particularly wide range of genera and species associated with them, such as sugarcane and rice, leading to difficulties for nematode control strategies. An excellent nematological asset for distribution maps and host-range data is available and updated regularly as a useful source for determining nematode damage potential (http://www.cabi.org/dmpd). Also, world food production for major food commodities and their main nematode pests of importance have been reported (Nicol *et al*., 2011). For example, *Meloidogyne* spp., *Pratylenchus* spp., *Heterodera* spp., *Punctodera chalcoensis*, *Paratrichodorus* spp. and *Longidorus breviannulatus* are the main PPN pests of maize (*Zea mays*), whose major producers are the USA, China and Mexico. Likewise: France, the USA and Australia are major producers of barely (*Hordeum vulgare*), infected by the main PPN *H. avenae*, *Meloidogyne* spp., *A. tritici* and *Pratylenchus* spp.; the USA, Nigeria and India major producers of sorghum (*Sorghum bicolor*) infected by *Belonolaimus longicaudatus*, *Paratrichodorus* spp., *Pratylenchus* spp. and *Criconemella* spp.; China, Russian Federation and India of potatoes (*Solanum tuberosum*) infected by *Globodera* spp., *Meloidogyne* spp., *N. aberrans*, *Pratylenchus* spp. and *Trichodorus* spp.; China, India and Indonesia of rice (*Oryza sativa*) infected mainly by *D. angustus*, *A. besseyi*, *Heterodera* spp., *Meloidogyne* spp., *Hirschmanniella* spp. and *Pratylenchus* spp.; and China, India and the USA of wheat (*Triticum aestivum*) infected mainly by *Heterodera* spp., *Pratylenchus* spp., *Meloidogyne* spp., *A. tritici* and *D. dipsaci*.

Contrary to resistant plants, PPN readily reproduce on susceptible plants and their final populations proportionally increase as their initial inoculation/population levels increase up to a certain limit. It is probable that nematode competition, at such a limit, per space unit of the roots, is so high that it usually adversely affects the infectivity, reproductive potential and final population of nematodes. Consequently, nematode rate of build-up often decreases as the inoculation level increases. In other cases, high PPN rates of build-up are observed especially after applying nematicides against some PPN. Such an unexpected increase in nematode numbers may be due to faulty application of nematicides, insufficient chemical and unfavourable weather conditions (Perry, 1953).

Luc *et al*. (2005a) gave detailed reflections on PPN distribution especially in subtropical and tropical, compared to temperate, agriculture. Askary *et al.* (2012) made an attempt to highlight the occurrence of PPN and their diversified nature of attack in temperate fruit crops, i.e. pome, stone and nuts. In temperate areas there are 'secondary species', but one main nematode parasite of a crop is often found that can be recognized easily and targeted for control. Such are not the cases for many tropical crops where numerous species of several different genera may be major parasites of a crop. One such example is sugarcane, which can be damaged by 10–20 different species of nematode belonging to the genera *Meloidogyne*, *Heterodera*, *Achlysiella*, *Pratylenchus*, *Xiphinema* and *Paratrichodorus*. The predictions of damage are usually difficult since component species of a nematode population may differ from one country to another. Such types of multispecies populations

have several consequences related to nematode control. First, the establishment of an effective crop rotation is hindered due to difference in the host status of each crop that depends on the species of nematode present. One such example is the Côte d'Ivoire, where *Crotalaria* was recommended as an intercrop for the control of *Meloidogyne* spp. on pineapple. The inter-cropping effect resulted in an effective control of the RKN but on the other hand an increase in the population of *Pratylenchus brachyurus* up to the level that was as harmful as *Meloidogyne* spp. Due to multispecies populations the search complexity for crop resistance to nematodes increases, and therefore targeting one nematode species for resistance no longer remains sufficient. A very good example for resistance to one species of nematode is the extensive planting of *G. rostochiensis*-resistant cultivars after the emergence of the PCN *Globodera pallida* takes place. A new and aggressive species of root-knot nematode, *M. floridensis*, should also be mentioned because it was not parasitized by the obligate bacterial parasite *Pasteuria penetrans*. The great variations in aggressiveness between the populations of burrowing nematode *R. similis* also affect future integrated pest management strategies. The basic facts of subtropical and tropical agriculture that differ from the temperate regions in the control of plant nematodes are the crops grown, the cultural practices and the farming systems. In subtropical and tropical agriculture, commercial or plantation crops are a common feature; however, the largest proportion of cultivated land in most of the tropical regions has smallholder farmers using traditional cropping practices. A wide range of crops is grown that covers grain, root, vegetable, cash and utility crops. The most common practice of cultivation is multiple or intercropping; however, monocropping is also practised. In the tropics, traditional agriculture is generally based on the reproduction of crops by vegetative propagation. This is not the case in temperate countries where the dependence is mainly upon seed-reproduced plants. Vegetative propagation can increase the dissemination of nematodes in plant tissues. The outstanding feature of traditional agriculture that creates a problem for nematologists is the complexity of the methods involved (Bridge, 1996). In contrast, modern farming in temperate countries is comparatively simple and the study and control of the nematodes is relatively easier. The nematode management methods that are used in subtropical and tropical countries differ slightly from those used in temperate countries; however, in practice they are more difficult to implement and need to be considerably modified in many circumstances; e.g. modern farms or plantations versus small rural farms with more traditional cultivation systems in developing countries. A modification in the existing agricultural practices is required in order to manage nematode populations and this is one of the most acceptable alternatives to chemical control for both the small- and large-scale farmers in the tropics. Crop rotation can vary from non-existent, where there is continuous cultivation of a susceptible crop or crops often planted sequentially in one year, through what can be termed random rotation, to a relatively sophisticated form of rotation. Good PPN-targeted practices lower the population densities of nematodes and their frequency of occurrence. However, most crop sequences have been designed to prevent disease outbreaks or increase available nutrients, and are not always compatible with nematode control. An effective control by crop rotation can be made by understanding the nematodes involved and the accepted cropping systems or some modifications in it. Many other cultural methods to manage PPN have been reported (Sikora *et al*., 2005). Resistant cultivars have brought a dramatic increase in the yield of several crops and thus also appear to solve many PPN problems, particularly with the work on gene transfer. However, in practice such cultivars mainly show resistance to only a limited number of nematode genera. These nematodes tend to belong to the groups of parasites, such as the Heteroderidae, which have a highly developed host–parasite relationship where cell modification occurs and is required for successful reproduction of the nematodes (Molinari, 2012). Management practices such as seed treatment with botanicals, chemicals and biological control agents (BCAs) has also been reported and is considered a safe, economic and effective method in the management of

RKNs (Askary, 2012). There are several major groups of migratory endoparasitic nematodes of subtropical and tropical areas that cause cell destruction without modifying the host tissues. These are the species belonging to the genera *Radopholus*, *Pratylenchus*, *Hirschmanniella*, *Scutellonema*, *Helicotylenchus* and *Hoplolaimus*. With one exception only, no true resistance has been found in banana cultivar against *R. similis*. However, where the possibility exists for nematodes such as *Heterodera*, *Meloidogyne* and *Rotylenchulus*, such research nevertheless remains aleatory and very costly. Continuous research for many years with expenditure of several million US dollars was necessary to obtain a soybean cultivar resistant to *H. glycines* (Luc *et al*., 2005a).

1.3.5 Progress in molecular and biochemical studies

There are several most important species that are obligate biotrophs and are difficult to culture in large numbers. PPN are difficult to handle as experimental organisms, especially on the molecular basis. However, a significant impact has been found with the new advance in genomics tools suitable for use with small quantities of starting material as well as the evolution of tactics such as RNA interference (RNAi) for use with many phytonematodes (i.e. Chen *et al*., 2005). Basically, numerous molecular studies are dedicated to resolve species determination and phylogenetic relationships by the sequencing of ribosomal DNA (Jones *et al*., 2013). To develop species-specific primers for quantitative polymerase chain reaction (qPCR), sequence information from ribosomal DNA has been used for identifying PPN in the field (e.g. Berry *et al*., 2008; Yan *et al*., 2012). Moreover, expressed sequence tag (EST) datasets are now available for a wide range of species (reviewed by Jacob and Mitreva, 2011), and the genome sequences of three species, i.e. *Meloidogyne incognita* (Abad *et al*., 2008), *M. hapla* (Opperman *et al*., 2008) and *B. xylophilus* (Kikuchi *et al*., 2011) have been published, with others in the pipeline (Jones *et al*., 2013). As more nematode genomes are sequenced, it will be interesting to determine the extent of peptide hormone mimicry among sedentary PPN and how the presence or absence of such mimics correlates with differences in feeding-cell ontogeny and host range since nematode-secreted peptides can function as molecular mimics of endogenous plant peptides to promote parasitism (Mitchum *et al*., 2012). Fortunately, the two most economically important biotrophic, sedentary endoparasitic groups, the cyst nematodes and RKNs, could infect *Arabidopsis thaliana* (Sijmons *et al*., 1991). Therefore, the resources developed with an aim to study this model plant are made available for the analysis of plant–nematode interactions. This allowed the mechanisms by which the complex feeding structures induced by the nematodes are produced to be tracked through the full scope of genomic resources (Jones *et al*., 2013).

Two molecular approaches have been field-tested to control a wide nematode range by either limiting use of their dietary protein uptake from the crop or preventing root invasion without a direct lethality (Atkinson *et al*., 2012). The former approach demonstrates the best-established strategy for developing genetically modified nematode-resistant plants which targets feeding nematodes and involves over-expression of cysteine proteinase inhibitors (cystatins) to interfere with intestinal digestion of their dietary protein intake from the plant. Cystatins have an important role against several nematodes differing in modes of parasitism, which includes results from field trials of transgenic potatoes infected by *Globodera* (Fuller *et al*., 2008). Since nematodes must sense and respond appropriately to a range of chemical signals in order to achieve a successful parasitic interaction, the second approach has distinct, synthetic peptides that can interfere with nematode chemoreception by binding to either acetylcholinesterase (AChE) or nicotinic acetylcholine receptors, both targets in the PPN cholinergic nervous system. Transgenic plants were subsequently developed that secrete the peptides from their roots. The AChE-inhibiting peptide suppressed the number of *H. schachtii* (beet cyst nematode) females by more than 80% that developed on *A. thaliana*, while it was almost 95% in the case of *G. pallida* as expressed in the root tips of potato plants (Lilley *et al*., 2011).

Marker-assisted selection (MAS) for nematode resistance was initiated where the isozyme marker associated with an acid phosphatase (Aps-1) tightly linked to the tomato *Mi* gene for resistance to *M. incognita*, *M. javanica* and *M. arenaria* (Rick and Fobes, 1974). Molecular markers, which are DNA sequences produced by different technologies, are considered powerful tools in MAS. These tools are being applied in some important breeding programmes for nematode resistance (Young and Mudge, 2002). DNA markers, especially simple sequence repeats (SSR) or microsatellites, closely linked to RKN resistance genes, are actively being produced for different crops (Hussey and Janssen, 2002). DNA markers are essential for producing genetic linkage maps, comparative mapping analysis, tagging target genes and MAS. Yet, in the first stages of screening for unknown resistance resources, the detection of readily evident phenotypic reactions is the economically preferred method since many of the available germplasm resources remain to be characterized with respect to nematode resistance (Starr and Roberts, 2004; Molinari and Abd-Elgawad, 2007). This characterization may involve, to increase the economic efficiency, the screening of core collections of accessions, as proposed by Holbrook *et al*. (2000). Molecular markers are so costly that its application is not feasible in the first identifications where resistant resources are not known. On the other hand, DNA markers play an important role when resistance has to be characterized in terms of genes involved and genetic linkage maps. Similarly, in selection processes wherein both molecular and phenotypic markers can successfully be applied, it should be considered that, generally, DNA technologies depend on costly investments in terms of consumables and equipment, besides requiring highly trained personnel. Biochemical markers are phenotypic markers that are based on proteins and/or enzymes associated with the nematode-resistance status or response. They are originally identified from isozyme bands appearing on the electrophoretic patterns of extracts from tissue having resistance genes. A recurrent change in enzyme activity was proposed as a marker for an incompatible response of tomato to RKNs (e.g. Molinari and Abd-Elgawad, 2007). The technique used in the screening of core collections of tomato for RKN resistance may be much more suitable, simple and rapid as compared to glasshouse nematode bioassays. The detection of catalase activity in roots of young tomato seedlings may be performed soon after a few days of nematode inoculation. The process is inexpensive and also does not require particular expertise and equipment. A potential economic improvement of the technique, relying on differences in the endogenous catalase activity between resistant and susceptible tomato but also in other tissues such as seeds or leaves, needs to be investigated. The challenges of current classical, as opposed to biochemical, approaches for rating host suitability for PPN and critical factors influencing phenotypic expression of resistance were reported (Abd-Elgawad and Molinari, 2008).

1.4 Impact of Phytonematodes on World Agriculture

1.4.1 Rationale and methodology

Admittedly, crop losses caused by nematodes are a function of the interaction among plants, nematodes and environmental factors. A number of biotic and abiotic factors play a vital role in determining the activities and population densities of nematodes and their consequent impact on crop performance. Certain factors like host status, soil temperature, soil moisture, soil structure, organic matter, fertility level, biological enemies, aggressiveness and virulence of nematode population are amenable to suitable modification/adaptation by alterations in cultural practices so as to shift the ecological resultant in favour of the plant and against the target nematode species (Gaur and Seshadri, 1986). With the available information on the effects of some of these factors, a number of basic mathematical models have been evolved to depict the nematode population–crop yield relations and population growth patterns. Since it is too difficult for the nematode to be eliminated, the overall goal is to keep the population density as low as possible. So, knowledge and provision of the

conditions that maintain large and diversified populations of BCA to preserve nematode-suppressive soil are important.

We have previously highlighted some obstacles hindering perfect estimates of crop losses caused by the nematodes. Moreover, information requirements for crop-loss assessment purposes must include estimates of crop distribution and value, pest distribution and average infestation level, and finally a damage function relating average infestation and crop yield. All these estimates are susceptible to error, and interaction effects among biological components should be considered (Koenning *et al*., 1999). Yet, studies on the impact of phytonematodes on world agriculture are essential because they can let people know how serious nematode problems can be worldwide. Such studies are also considered the basis for nematode-management options. Reliable crop-loss estimates are important for establishing research, extension and budget priority (McSorley *et al*., 1987). Pest-specific crop-loss information is needed by government agencies, corporations involved with crop protection and production, and university systems for descriptive and predictive purposes (Noling, 1987). Regulatory policy action, pesticide impact assessment, resource allocation and programme prioritization are usually contingent upon crop-loss data. On-farm pest management decisions depend on anticipated crop losses and pest control costs (Ferris and Noling, 1987). Therefore, one should not be discouraged by some cautious tones and limitations in establishing such estimates.

Although most administrators agree that crop-loss estimates are important to justify public expenditure for research and education programmes in nematology, increasingly the sources of such information are unavailable. Koenning *et al.* (1999) gave some details for this issue. The 1987 bibliography relied on the pesticide impact assessment programme conducted by the US Department of Agriculture (USDA) in the 1980s. Pesticide impact assessment continues, but the form of data collection and publication changes in response to perceived needs of the reporting agency to supply information relevant to particular issues. So, the proposed ban on methyl bromide (MB) and other pesticides has resulted in economic analysis of alternatives. Michigan and North Carolina maintained comprehensive crop-loss estimates in response to disease and/or nematodes, but such publications have since been discontinued due to funding constraints. Likewise, groups working with particular commodities periodically or annually had developed estimates, but these efforts on some commodities, e.g. tobacco and groundnuts, have been discontinued.

An appropriate test of the value of PPN controls lies in doing the cost–benefit analysis. The choice criterion in cost–benefit analysis is the cost–benefit ratio (Khan, 1972). This ratio was implemented to demonstrate the benefits of PPN quarantine and certification programmes of Florida citrus (Inserra *et al*., 2005) and to analyse similar proposed programmes in Egypt (Abd-Elgawad and McSorley, 2009). In yield loss investigations, two main methods have been employed to assess losses: (i) trials of crops with and without nematicides can be quite informative; PPN populations have been reduced with nematicides and subsequent yields monitored; and (ii) the relations between PPN infestation levels and yields have been examined. The latter may be conducted through experiments aiming at establishing different levels of nematode-initial population (P_i) densities by cultivation of crops with different hosting capacities allowed to determine the economic threshold level. Both approaches have limitations (Duncan, 2005). Chemical nematicides may affect other fauna and flora associated with the tree in addition to nematodes. They may also directly affect plant development negatively (Cohn *et al*., 1968; Timmer, 1977) or positively (Wheaton *et al*., 1985). Relating yields to infestation levels can be confounded by unmeasured edaphic variables that affect both nematode and plant. Hence, researchers should already be aware of and consider all the factors affecting crop yields so that they can accurately determine crop losses due to PPN. During compilation of nematode losses from different experiments/sites, they should take care of, and standardize to compare, such factors as inherent differences in environment, plant genotype, experimental design, PPN species, variation in sampling protocols, extraction procedures, spatial distribution and calculation. For example, some

report damage as percentage yield loss and others as tonnes per hectare or as percentage yield gained after nematicidal application or as correlations of yield gains with declining PPN level. It is difficult to compare the damage threshold of the CCN from different studies because very few studies are truly comparable (Rivoal and Nicol, 2009). A significant consideration, often overlooked, is how to measure P_i. Usually it is counted as nematode numbers per gram of soil. A more appropriate measure is per unit volume of soil in order to allow for bulk density differences. Numbers per gram of root is probably the most appropriate, but is hard to count since it is often changing. It becomes remarkable when trying to relate results from tests where root densities are very different, e.g. pot versus field trials (http://www.fao.org/docrep/v9978e/v9978e07.htm). Nematode numbers per gram of root per unit of soil might also be appropriate. On the other hand, progress has been achieved to construct damage functions, conduct surveys on the distribution of PPN and develop methodology for obtaining loss estimates (Duncan and Noling, 1998). References regarding publications on estimation of losses and nematode surveys are included for each commodity, where available. Compilation of PPN losses rely heavily on the analysis-based opinions of well identified, with extensive knowledge, experts. For assessing yield loss, the criteria used may also comprise grower interviews, visual assessment based on foliage growth (necrotic, chlorotic, stunted and wilted plants), root symptoms and educated guess to expert opinions. Such interviews may include crop condition, qualitative and quantitative yield losses based on market value and lifespan of the crop (Anwar and McKenry, 2012). Yet, the relative economic impact of each PPN species should be upgraded from time to time since it is subjected to change with time as nematological research progresses and new species with their potential damage are discovered. Singh *et al*. (2013) stressed that new species are being described regularly, and species new to science have been found during phytosanitary inspections (e.g. *Radopholus bridgei* and *Meloidogyne thailandica*, both described from material intercepted in quarantine). One can easily recognize such a change when the importance of PPN in a relatively recent report is compared with older ones or even when the importance of a nematode species is changed with time.

1.4.2 Previous estimates of nematode damage

It is useful first to retrieve some previous estimates because of their importance in terms of being based on extensive questionnaires of experts in the field, provide periodic information about nematode losses, and can be compared with current estimates to see magnitude of the change over time, if any. Prior to 1987, only one crop-loss assessment related to PPN had been published by Feldmesser (1971), who estimated damage caused by PPN on 24 vegetable crops in the USA to be 11% and losses due to nematodes in 1967–1968 in the USA were approximately US$1.5 billion. Poinar (1983) indicated that PPN can ruin as much as 15% of each year's agricultural crop in the USA. Most plants can tolerate these parasites and their damage because they have achieved a state of equilibrium as the host. When this balance is upset, large scale damage to crops is seen. Later, an outstanding document on US crops and estimated yield losses caused by PPN became available (McSorley *et al*., 1987). This US bibliography relied on contacting one or more scientists in all 50 states as well as published reports that in most instances were based on survey information. Sasser and Freckman (1987) reported comprehensive crop-loss estimates due to PPN for selected crops on a worldwide basis, since IMP covered many countries. These estimates of world crop losses were based on survey data collected for that purpose. They estimated overall average annual yield loss on the world's major crops due to damage by PPN as 12.3%. According to Wittwer (1981), the estimation of annual yield loss was 10.7% for the 20 life-sustaining crops, i.e. banana, barley, cassava, chickpea, coconut, maize, field bean, millet, oat, groundnut, pigeonpea, potato, rice, rye, sorghum, soybean, sugarbeet, sugarcane, sweet potato and wheat. Other

economically important crops for food or export value, i.e. cacao, citrus, coffee, cotton, cowpea, aubergine, forages, grape, guava, melons, okra, ornamentals, papaya, pepper, pineapple, tea, tobacco, tomato and yam were reported to have an estimated annual yield loss of 14%. Collectively, the overall average was 12.3%. Losses for the above-mentioned crops in developed countries averaged 8.8% compared with 14.6% for developing countries. Worldwide crop losses due to nematodes on 21 crops, 15 of which are 'life sustaining', were estimated at US$77 billion annually; the US portion alone was US$5.8 billion. They concluded that the real figure, when all crops throughout the world are considered, probably exceeds US$100 billion annually.

Generally, many publications cite a 1987 international opinion survey of 371 nematologists to support a staggering yield loss by PPN of US$78–125 billion worldwide annually. McCarter (2008) commented on the 1987 report of Sasser and Freckman as a reflection of informed opinion but not necessarily field data, and so reliability of these estimates remains difficult to assess. In the USA, other surveys of opinion have been carried out, and detailed information is available for some geographies and crops. A survey of 35 states on various crops, including maize, cotton, soybean, groundnut, wheat, rice, sugarcane, sorghum, tobacco, numerous vegetable crops, fruit and nut crops and golf greens indicated PPN-derived loss of up to 25% (Koenning *et al*., 1999). Their data were reported systematically by state and included the assessed loss, acreage of production, source of information, PPN species or taxon when available, and crop value. Handoo (1998) estimated global crop losses due to nematode attack in the region of US$80 billion. In order to illustrate the overwhelming effect of PPN in terms we can understand, it is estimated that US$200 million worth of just wheat crops are lost every year in Australia alone due to roundworms (Hodda, 2004). Initially, for better understanding of the value-capture proposition for nematode control, McCarter (2008) used the 1987 international loss estimates by crop (Sasser and Freckman, 1987) on 2001 data for crops and value by country including yield and currency exchange rates. He found that the extrapolated 2001 loss for the 40 most substantial crops in the survey totalled US$118 billion (11% of production). The decrease in value of the dollar relative to some other currencies and the increase in commodity prices since 2001 may inflate this estimate further. Non-food plants including ornamentals, turf and forest trees (e.g. pine) were not included in this damage assessment. He stressed that the representation of loss by crop is more significant than the total estimated damage. Moreover, given the more subtle effects of low infestation levels, such figures are probably a vast underestimate (Nicol *et al*., 2011). Chaudhary *et al*. (2011) reported that about 70% of the damage caused by PPN is attributed to RKNs, which infect a wide range of crops, particularly vegetables, and cause losses up to 80% in heavily infested fields with average yield losses ranging from 28 to 68%. Atkinson *et al*. (2012) reported losses induced by PPN to be US$118 billion worldwide. Singh *et al*. (2013) stressed that yield loss data is difficult to obtain because of the complex interactions of plants, nematodes, other soil organisms and soils. They presented a systematic list of PPN species of phytosanitary importance to provide useful preliminary information and guidance to nematologists in many countries assessing the risks from PPN. Their suggested criteria for assessment can also prove helpful in pest risk analysis and prompt appropriate gathering of additional data, thus enhancing global biosecurity. Substantial crop losses due to PPN could be much greater if the damage to crops that is still localized became widespread. Several species of PPN having a low impact locally can cause much greater impact when introduced to new areas.

1.4.3 Our current estimates

The existing paradigm for estimates of yield losses caused by PPN has become old. We attempted to update such losses; yet, due to non-available funds to help in collecting and compiling these losses, our questionnaire in 2013 went almost like an echo for many countries. However, 27 nematologists have responded

from 14 countries (Tables 1.3 and 1.4). It is worth mentioning that our questionnaire has no recent competitor due to different reasons. Some nematologists attempted to obtain damage figures for PPN worldwide but the gathered information was far too speculative to be of use for publishing. However, the need for updated research of such yield losses to get, as best we can, reliable estimation as the basis to establish significant nematological issues like research, extension and budget priority is apparent.

Since we were cautious in using the questionnaire results, we drew upon both Sasser and Freckman (1987)'s report and surfing the internet for damage caused by phytonematodes as well. When the range of loss for a specific crop was so wide and the estimated figure of Sasser and Freckman (1987) was the median, or almost in the centre of that range, we selected such a figure. In other words, although the arithmetic mean is the most suitable average to measure the central value of the loss distribution, we relied upon the weighted mean supported by 371 responses from IMP for 20 life-sustaining (Table 1.3) and 20 economically important (Table 1.4) crops. Accordingly, for the crops (Table 1.3) that stand between man and starvation (Wittwer, 1981), average worldwide annual yield losses due to nematodes have been estimated as 12.6%, which equalled to US$215.77 billion on a monetary basis. The crops that represent a miscellaneous group important for food or export value are reported (Table 1.4) to have an average annual yield loss of 14.45%, which totalled to US$142.47 billion. Losses for the total 40 crops (Tables 1.3 and 1.4) averaged 13.5%. Worldwide crop losses due to nematodes on 37 crops are estimated at US$358.24 billion annually based on 2010–2013 production figures and prices (Tables 1.3 and 1.4). These figures are staggering, and the real figure, when all crops throughout the world are considered, probably exceeds such estimations. Our current estimates far exceed previous ones (e.g. Sasser and Freckman, 1987), probably because of challenging issues which aggravated PPN losses. In Table 1.4, the crops selected by virtue of their production value on a world basis or their importance in world trade may largely be regarded in this context as major crops among the long list of cultivated commodities worldwide. Yet, some accurate data for forages, guava and ornamentals were not available (Table 1.4). Specific examples of the figures obtained from our current questionnaire that may reflect the reality of the situation, and may be of use for locally-oriented purposes, are as follows: yield losses caused by PPN were in banana (30%), cassava (10%), coconut and guava (10–35%), maize (20%), millet (15%), groundnut (10–20%), potato (20–35%), sorghum (10–40%), soybean (15–25%), sugarcane and tobacco (10–15%), citrus (10–30%), coffee (10–20%), cotton (8–18%), aubergine (25%), forages, tomato and grape (20–30%), melons (20%), okra (35%), papaya (5–15%), pepper and yam (5–10%) in Brazil (P.R.P. Martinelli, Jaboticabal-SP, Brazil, 2013, personal communication); banana (1–4%), maize (1–3%), potato (2–10%), sugarbeet (4–8%), wheat (1–4%), citrus (2–5%), cotton (1–4%), aubergine (4–10%), grape (7–10%), melon (6–15%), okra and pepper (5–15%), tobacco (7–15%) and tomato (8–20%) in Greece (I. Vagelas, Technological Educational Institute of Thessaly, Department of Plant Production, Greece, 2013, personal communication); banana (26.4–45.4%), field bean (37.1–45.2%), rice (25.5–61.6%), soybean (35.2–50%), aubergine (37.1–44.2%), melons (14.3–60.5%) and tomato (37.5–96.1%) in the Philippines (C. Cumagun, Philippines, 2013, personal communication); lucerne (5–10%), barley (25–30%), cucumber (30–50% up to 80%), oat (5–10% up to 60–70%), onion (up to 50%), potato (2–70%), rice (up to 75%), rye (5–7%), soybean (33–58%), strawberry (54–86%), sugarbeet (5–10% up to 40–50%), tomato (30–50% up to 80%), wheat (5–10% up to 30%) in the Russian Federation (M. Pridannikov, Moscow, 2013, personal communication); banana, forages, soybean and sugarbeet (10–25%), barley (0–5%), chickpea and sorghum (0–10%), maize and field bean (5–15%), groundnut (15–50%), potato and ornamentals (0–20%), rice and papaya (0–25%), sugarcane (10–30%), sweet potato (5–15%), wheat (0–15%), citrus (15–65%), cotton (5–25%), cowpea (10–50%), aubergine (10–30%), grape (15–75%), guava (0–25%), melons (0–45%), okra (10–60%), pepper (0–35%) and tomato (20–80%) in Egypt (M.F.M. Eissa, The National Research Centre (Cairo), 2013, personal communication); maize (0–7%), field bean (8%), oat (8%), groundnut (1–5.5%), potato

Table 1.3. Summary of estimated annual yield losses in life-sustaining crops due to damage by plant parasitic nematodes – world basis (updated[a] and adapted from Sasser and Freckman, 1987).

Life-sustaining crops	Loss (%)	Actual production (1,000 t)	Price (US$/t)	Actual loss in production (1,000 t)	Loss (US$ million)
Banana	21.3	99,996.5	937.6	27,063.86	25,375.07
Barley	6.1	134,000.0	194.7	8,705.01	1,694.86
Cassava	10.0	250,000.0	509.2	27,777.78	5,408.33
Chickpea	13.7	10,918.0	720.0	1,733.22	1,247.92
Coconut	20.7	62,451.0	1,500.0	16,301.84	24,452.76
Field bean	12.5	12,000.0	210.0	1,714.29	360.0
Groundnut	14.2	37,643.0	2,800.0	6,229.96	17,443.89
Maize	7.9	844,405.0	234.9	72,429.96	17,013.8
Millet	12.0	36,000.0	490.0	4,909.09	2,405.46
Oat	13.5	21,300.0	255.3	3,324.28	848.69
Pigeonpea	13.2	3,680.0	278.0	559.63	155.58
Potato	15.1	330,047.0	220.0	58,700.94	12,914.21
Rice	12.8	672,015.0	503.8	98,644.40	49,697.05
Rye	3.5	12,328.0	303.0	447.13	135.48
Sorghum	7.8	65,000.0	220.6	5,498.92	1,213.06
Soybean	13.1	269,000.0	429.4	40,551.21	17,412.69
Sugarbeet	11.8	228,452.0	67.8	33,534.24	2,273.62
Sugarcane	24.9	1685,444.0	35.4	558,822.31	19,782.31
Sweet potato	10.2	106,569.0	80.0	12,104.72	968.38
Wheat	7.0	650,881.0	305.5	48,991.04	14,966.76
Total US$					215,769.92

[a]Information based on 2010–2013 production figures and prices and additional worldwide survey of 27 nematologists in 2013.

Table 1.4. Summary of estimated annual yield losses for economically important crops due to damage by plant parasitic nematodes – world basis (updated[a] and adapted from Sasser and Freckman, 1987).

Economically important crops	Loss (%)	Actual production (1,000 t)	Price (US$/t)	Actual loss in production (1,000 t)	Loss (US$ million)
Aubergine	18.1	43,174.0	3,190.0	9,541.51	30,437.41
Cacao	10.5	3,986.0	2,483.6	467.63	1,161.41
Citrus	14.2	11,763.0	290.0	1,946.79	564.57
Coffee	15.0	8,359.4	3,400.0	1,475.19	5,015.64
Cotton	11.1	27,400.0	2,380.0	3,421.15	8,142.33
Cowpea	15.1	42,000.0	1,000.0	7,469.97	7,469.97
Cucumber	18.5	417,000.0	733.0	94,656.44	69,383.17
Forages	9.1	–	–	–	–
Grape	14.8	67,322.2	508.0	11,694.47	5,940.79
Guava	11.9	–	–	–	–
Melons	15.7	25,000.0	310.0	4,655.99	1,443.36
Okra	21.3	7,896.2	5,610.0	2,137.09	11,989.08
Ornamentals	11.1	–	–	–	–
Papaya	15.1	11,223.0	287.6	1,996.08	574.07
Pepper	12.5	320.0	7,700.0	45.71	352.00
Pineapple	14.9	19,412.9	1,140.0	3,398.97	3,874.82
Tea	8.2	4,700.0	8,140.0	419.83	3,417.38
Tobacco	11.8	7,113.0	2,820.0	951.63	2,683.58
Tomato	22.4	150,513.8	2,230.0	43,447.28	96,887.44
Yams	17.7	48,701.0	690.4	10,473.97	7,227.04
Total US$					142,473.8

[a]Information based on 2010–2013 production figures and prices and additional worldwide survey of 27 nematologists in 2013.

(1.2–12%), rye (3%), soybean (8%), sugarbeet (5%), sweet potato (5.2%), wheat (4%), cotton (1.6%), cowpea and aubergine (3.5%), forages, lucerne and clover (1–5%), grape (12–20%), melons (10%), cucumber (1–12%), okra (5.2%), pepper (7%), tobacco (0.9%) and tomato (2–3%) in Florida, USA (R. McSorley, Florida, 2013, personal communication); barley, oat, wheat and cotton (5%), field bean (6%), potato, sweet potato, sugarbeet, melons, ornamentals and yam (10%), citrus, aubergine, grape and tomato (15%), forages (8%) and pepper (11%) in California, USA (Becky Westerdahl, California, 2013, personal communication); banana (7.9–34.6%), barley (70.2%), betelvine (4–50%), brinjal (16.6%), carrot and cucurbits (18.2%), castor (13.9%), chickpea (18.3%), chili (12.9%), maize (17–29%), mungbean and urdbean (8.9%), citrus (6.8–17.5%), cotton (18–32%), cowpea (27.3%), jute (12–54.4%), groundnut (21.6%), rice (10.5%), tomato (27.2%), grape (11–28%), melons (20%), okra (14.1%), pigeonpea (12.6%), sesame (4.4%), spices (38.5–64.6%) and wheat (32.4–66.6%) in India (Khan *et al*., 2010); banana (10–25%), barley (10–20%), oat (10–15%), groundnut (5–20%), potato (5–10%), rice (5–15%), soybean (5–25%), sweet potato, melons, aubergine and wheat (5–30%), ornamentals (5–10%), tobacco (5–30%) and tomato (5–35%) in China (Heng Jian, China Agricultural University, Beijing, 2013, personal communication).

Additional information on cost figures may provide a background of the current estimates. For example, the University of Florida nematode assay laboratory charges US$20 per sample for soil/root assays and conducts about 3000 samples per year, 90% are from turf grasses, 5% from ornamentals and the remaining 5% split among various agronomic, vegetable and fruit crops (W.T. Crow, Florida, 2013, personal communication). Differences in commodity, nematicides/control method and sampling prices among countries should also be considered if a feasibility study is established for combating nematode pests in a specific region. For example, in a developing country like Egypt, an equivalent of US$20 per sample is charged for soil/root assays with five samples/Faddan (Faddan = 4200 m^2). The prices also depend on number of samples; if the number of samples increases, the price decreases up to 70% discount. Prices for analyses of samples on quarantine and economically important nematodes in Serbia vary (Violeta Oro, Serbia, 2013, personal communication): presence of *G. pallida* and *G. rostochiensis* – ca. US$20/sample; presence of *G. pallida* and *G. rostochiensis*, *M. chitwoodi*, *M. fallax* and *D. destructor* – ca. US$50/sample; but the prices decrease up to 50% when samples increase. Since vegetables are the most susceptible host for RKNs and their infestations on tomato are common in Africa and worldwide (Netscher and Sikora, 1990; Agbenin, 2011), we can give an example as a rough estimation for loss value of Egyptian tomato. In Egypt, tomato was planted at 242,851 Faddan in the old and 272,374 Faddan in the newly reclaimed land with average production of 17.152 and 16.175 tons/Faddan, respectively, in the year 2012 (Anonymous, 2012b). Governmental recommendations adopted to combat RKN species in tomato fields include the use of one of the following nematicides: Rugby, Vydate, finatode, and Nemathorin. We assume that tomato losses caused by *Meloidogyne* spp. are confined only to 170,000 Faddan because the nematodes seldom damage tomato both in the winter season and in the old land with clay soil (Abd-Elgawad and Aboul-Eid, 2005). Therefore the actual and potential production of *Meloidogyne* spp.-damaged areas were 2,749,750 t and 5,499,500 t of tomato, respectively. Assuming 50% average losses by the nematodes (M.F.M. Eissa, The National Research Centre (Cairo), 2013, personal communication), then 2,749,750 t of tomato equivalent to US$824,925,000 (1 t of tomato = US$300) are the losses due to *Meloidogyne* spp. only in the truncated area. As for costs to treat such a tomato hectarage using Egyptian formal recommendations (Anonymous, 2012a), i.e. 3 l of Vydate/Faddan to be sprayed on tomato plants twice (total 6 l Faddan; 1 l Vydate = US$26) when plants are not treated during seedling stage in a healthy nursery soil (6 × US$26 × 170,000 = US$26,520,000); labour and application costs (US$3 × 170,000 = US$510,000); and labour, transportation and sampling costs (US$40 × 170,000 = US$6,800,000). So, net gain of nematicide treatment will exceed US$791 million (824,925,000–26,520,000

–510,000–6,800,000 = US$791,095,000). In China, RKNs (*M. incognita*, *M. hapla*, *M. arenaria* and *M. javanica*) cause yield reduction of vegetables at 10–20%, 30–50% normally or 70% yield loss at high RKN populations; heavy RKN populations cause plants to die early and they have to re-transplant several times in one season; estimation of loss by RKN is over US$0.4 billion annually. *Bursaphelenchus xylophilus* caused only 265 trees to die in 1982 in Nanjing, Jiangsu province. Nowadays, *B. xylophilus* has been found in 192 areas of 14 provinces in China and responsible for the loss of 17 million steres of softwood chips per year. Therefore it was estimated to have killed more than 500 million pine trees. *Heterodera avenae* is a major limiting factor in wheat production, infesting over 3 million ha in China. In Henan province, the area was 1.3 million ha and the yield losses were up to 28.8–35.6%. Hectarage of damage by *H. glycines* is over 2 million ha/year causing yield loss of about 20–50%, which in monetary term is about US$1 billion (Heng Jian, China Agricultural University, Beijing, 2013, personal communication).

1.4.4 The true cost of nematodes

As the Arabic proverb says, 'things are clearly seen in the presence of their antitheses'; indirect yield losses also generally remain unaccounted for in most loss figures. First, it should be stressed that the nematode loss data based on comprehensive surveys and experiments are more accurate and reliable than estimates based upon the guesses of nematologists/experts. The latter may be much different from having survey data from a number of fields in the state. Second, let us consider root-knot on tobacco, which could easily make a loss of 20% to a tobacco field as an example. Nematologists, crop advisors, growers and others see this and are very impressed; they know that root-knot can do this bad damage and they remember it. So, when asked about losses in tobacco, they will recall this situation and give that high number. But the key point is that most tobacco fields will not have these kinds of losses. So, the survey is needed to average the field with 20% loss with fields that have low or zero losses, to get a true impression of the statement. For tobacco (and many other crops) in the USA, most fields will show little or no losses from nematodes because the growers are aware of the problem and manage for it with nematicides, rotation, etc. Contrary to developing countries, in the USA a grower that had a 20% loss might go out of business if such losses were typical. The same is true with tomato and many other crops; losses are quite low because nematode management is a routine practice on these crops in the USA. In a few cases, we can get an idea of potential losses if someone had made an experiment with management treatment versus untreated control. There were data from Iowa state in nematicide and untreated plots showing that losses in the control plots were quite high (McSorley *et al.*, 1987). But for the most part, these are potential losses, not true crop losses, because most of the growers use nematicides to avoid these losses. On the other hand, where nematodes are not managed (many growers are unaware of nematodes, so they will run into problems with them) losses due to nematodes will be quite high. But in those places where nematodes are always managed, much of these losses can be prevented. Putting those two things together, one will emphasize and make a strong case for the importance of nematode management! Openly, often the presence of nematologists or farm advisors is sufficient to help these growers know about nematodes so that they can carry out some management and minimize future losses. Third, it is very difficult to keep track of indirect monetary losses. If we see a place where nematodes are not managed much and cause significant losses (20%), we could estimate how much money would be lost to nematodes based on the value of that crop. On the other hand, if we see a fumigated tomato field where the loss to nematodes is only 1%, that would be a small direct monetary loss, but one would also have to add indirect costs because the grower would spend money on the fumigant and its application. It is hard to obtain data on these things to find out the true cost of nematodes to an economy. Unfortunately, sometimes governments and administrators do not recognize the importance and value of nematology. Rather than spend some thousands of

dollars to hire a nematologist, they prefer to save that money to keep the budget low. But they do not think that the economy may lose many millions of dollars in crop losses because the nematologist is not there to advise and help the growers. Fourth, losses from PPN may surpass agriculture to another sector(s), e.g. tourism. *B. xylophilus* in China does not kill pine trees only, but also affects the beautiful scenery of the tourist resort of the famous Yellow Mountain in Anhui province. Yellow Mountain's classic attractions could be counted as five natural wonders in the winter: the imaginatively named pines, oddly-shaped rocks, sea of clouds, hot springs and winter snowcaps. So, there will be catastrophic loss if pine trees die at Yellow Mountain.

The degree of damage a nematode causes can also be dependent upon host and age. Admittedly, prevailing soil property, environmental and climatic status all have an impact on the threshold population density, above which measurable damage takes place.

Several factors have an influence on yield losses such as the pathogenicity of the nematode species involved, population level of nematode at planting stage, susceptibility and tolerance of the host and range of environmental factors. For example, *Tylenchorhynchus martini* causes damage on sugarcane at populations between 600 and 6400/plant, whereas on onions only five individuals of *P. penetrans* per seedling will cause serious damage (http://www.enclyclopedialive.com). Hence, models usually assess such losses as proportions of the nematode-free yield (http://www.fao.org/docrep/v9978e/v9978e07.htm). So, variations in these estimates are not surprising. Some of these data come from surveys in individual counties, governorates or even states, others come from nematicidal trials or through the relationship between natural nematode infestation levels and yield, and some are just educated guesses. But they give our best impression of these losses.

Numerous nematologists (e.g. McCarter, 2008; Nicol *et al*., 2011) advocated that surveys of crop losses are likely to be a fair underestimate of the true figure, as many growers, particularly in developing nations, are still unaware of PPN. This is mainly because PPN are usually small and mostly live hidden in soil, under water, or in the plants, colourless, and the symptoms they cause are often nonspecific. Moreover, other crops that are vulnerable to PPN damage have not been considered in calculating the current global losses from PPN. For example, the above-mentioned nematode losses of betelvine, mungbean and urdbean, sesame, jute and spices in India were not considered (Tables 1.3 and 1.4). Also, lack of published information on the pathogenicity and economic importance of some less well-known PPN species causes difficulty in predicting the potential impacts of these species. Although more than 100 valid species are known from PPN genera *Xiphinema*, *Tylenchorhynchus* and *Hemicycliophora*, economic importance of only few species from these genera has been investigated (Singh *et al*., 2013). The lack of publicly available documentation on pest risk analysis from different countries of the world and paucity of transparency in the process leads to differences in how the phytosanitary importance of species is rated. For instance, *B. mucronatus* is considered of phytosanitary importance in some countries, but not in others. This is partially due to uncertainty on whether the species can cause disease. On the basis of research conducted in recent years it has been demonstrated that this species can carry pathogenic bacteria (Zhao *et al*., 2009; Singh *et al*., 2013).

Another aspect to be considered while assessing costs of phytosanitary risks is intraspecific variation. This is generally ignored because most phytosanitary regulation is on the taxonomic level of the species (Singh *et al*., 2013). However, species from the above-mentioned economically important genera have pathotypes/races with distinctive host responses and differences in host range. Under the International Plant Protection Convention (IPPC), absence of pathotypes in a country is justification for implementation of quarantine measures against exotic pathotypes (FAO, 2011; EFSA (Panel on Plant Health), 2012; Singh *et al*., 2013). In the USA, potato pathotype of *N. aberrans* is absent and only the sugarbeet pathotype of *N. aberrans* is present, and therefore potato pathotype is a regulated pest in USA. The highly damaging species such as *G. pallida*, *G. rostochiensis*, *H. avenae* and *D. dipsaci*, which have many pathotypes, may require further phytosanitary

risk assessment of pathotypes in order to prevent their spread and justify regulatory measures. It is unclear how widespread pathotype variability is present in many of the PPNs which are less well investigated. However, if it is as common as in the best known species, then this is another reason to take the same broad approach. A major hindrance in the efficacy of newly introduced resistant cultivars is the selection of PPN pathotypes/races that are able to break down the resistance (Luc *et al*., 2005a). The existence of resistance-breaking pathotypes is a major problem in breeding programmes in temperate crops. Similar complications are also expected during breeding of resistant cultivars for tropical crops. In the case of availability of resistant cultivars suitable for the conditions prevailing in a country, many other factors are to be taken into consideration before their successful introduction. For example, subsistence farmers are not aware of the fact that resistance caused by *Mi* gene breaks down at high temperatures or that nematode-resistant tissue culture banana plantlets are still susceptible to damage while in the seedling stage. A marked contrast is expected in what can be achieved with the big producer compared with the rural farmer; however, local needs have to be taken into consideration. For example, when dwarf rice cultivars were introduced to prevent lodging (Mydral, 1974), people in South-east Asia were deprived of their normal source of rice straw that they used as animal feed, bedding and thatching material. In the past few years development in the field of transgenic plants with resistance to insects, detection of genes in the plant that are responsible for giant cell formation and genes in plants required for protein synthesis by the nematodes may result in new forms of resistance. The cost of developing transgenic crops is enormous but presumably their long awaited marketing is about to occur.

1.5 Challenging Issues Related to the Estimates

Staggering though the estimates were as reported by Sasser and Freckman (1987) or herein (Tables 1.3 and 1.4) for the high nematode losses, further unexpected bad effects have been occurring in a way that may aggravate such losses. Such factors include reduced number of effective nematicides available and limitation in their use due to environmental issues, increased adoption of intensive agriculture, climate change, occurrence of resistance-breaking PPN pathotypes on economically important crops, and potential introduction of quarantine nematodes especially with plant propagation materials, to name but a few.

1.5.1 Redirections in the type and choice of applicable nematicides

Since the 1960s, most of the vegetable production in many parts of the world such as Florida and California in the USA has been fumigated with MB. So, growers just ignored nematodes, reckoning on the good effects of MB for nematode control, even though their fields were severely infested. Now they must grow crops in these infested sites without MB since it was banned as mentioned above. Non-fumigant granular and/or liquid formulations of contact and/or systemic nematicides are often not as effective as fumigants in increasing yields because they do not have broad-spectrum activity and in most cases only inactivate nematodes for short periods of time (Sikora *et al*., 2005). So, in the next few years, without an efficient alternative to MB, absence of such an effective fumigant will become a major crisis, particularly in crops such as tomato, pepper, strawberry, cucurbits, other vegetables and field-grown ornamentals, which have relied on fumigation in the past. The loss estimates for some of these crops were relatively low (Sasser and Freckman, 1987), because in 1987 MB was available and widely used. In the coming years we will be seeing losses without MB-effective control. Moreover, nematicides such as organophosphates and carbamates are non-specific neurotoxins with poor environmental and worker-safety profiles. Many of these nematicides have been banned, withdrawn from the market, or are under a re-evaluation process. For example Temik (aldicarb), a carbamate insecticide, is a commonly used commercial

nematicide. It is important in potato production, where it has been used to control soilborne nematodes. Aldicarb is a cholinesterase inhibitor which prevents the breakdown of acetylcholine in the synapse. In case of severe poisoning, the victim dies of respiratory failure. It is no longer authorized for use in the European Union (EU) and in August, 2010, Bayer Crop Science announced that it planned to discontinue aldicarb by 2014 (http://en.wikipedia.org/wiki/Nematicide). 1,2-Dibromo-3-chloropropane (DBCP) was an effective nematicide that also tragically caused human sterility, but it was banned in the late 1970s. Except for abamectins, which are used as seed treatments (e.g. Rich and Kavitha, 2006), no new class of effective nematicidal chemistry has been commercialized since the 1970s (McCarter, 2008). The future of nematicides for phytonematode control will depend on the formulation of new compounds that are both effective as well as environmentally safe. Moreover, advances in application technology, such as treatment by seed coating or application of chemicals through chemigation and development of systemic nematicides that move basipetally, are urgently needed.

1.5.2 Intensive agriculture system

This system uses high levels of complementary inputs, e.g. fertilizers and pesticides, to achieve maximum yields at the lowest possible cost from the same cultivated area. Indeed, the list of negative effects of intensive farming seems to be getting longer: soil degradation, salination of irrigated areas, over-extraction and pollution of groundwater, resistance to pesticides including nematicides, erosion of biodiversity, etc. (http://www.euractiv.com/cap/intensive-crop-production-linksdossier-506029). In Florida, the intensive citriculture system suppressed entomo-nematodes and consequently had the potential to exacerbate herbivory by insect pests (Campos-Herrera *et al*., 2013b).

1.5.3 Climate change

The most likely consequences of climate change are shifts in the geographical distribution of plant host and pathogen and altered crop losses, caused in part by changes in the efficacy of control strategies (Coakley *et al*., 1999). Such a change does not exclude the threat of the emergence of new pests, including nematodes (Nicol *et al*., 2011). Carter *et al*. (1996) predicted the increase of RKNs as climate changes because of additional pathogen generations per year in a warmer climate. This is especially important since most nematode life processes have thermic optima that determine the ideal geographic ranges of nematodes (Luc *et al*., 2005a). Boag *et al*. (1991) used data from soil samples collected during the European PPN Survey to assess the possible impacts of climate warming on the geographical range of virus-vector nematodes. Initial analyses of nematode presence–absence data suggested a close association between mean July soil temperature and nematode distribution. Based on this result, the authors predicted that climate change could result in increased nematode and virus problems in northern Europe; they estimated that a 10°C warming would allow the nematode species in the study to migrate northward by 160 to 200 km (Neilson and Boag, 1996). Although nematodes migrate very slowly, humans are credited with efficiently disseminating them. Nematode spread into new regions could put a wide range of crops at risk; additionally, introduction of new crops into a region could also expose them to infestation by nematode species already present. Changes in precipitation could influence nematode distribution on a large scale, although previous findings had suggested that soil moisture would not affect nematode distribution in most agricultural soils in northern Europe (Boag *et al*., 1991; Neilson and Boag, 1996; Coakley *et al*., 1999). Yet, in an alternative example, the rice RKN, *M. graminicola*, can be kept under damaging levels through good water management. However, the reduced availability of water following climatic changes and/or competition for urban use, reduced quality of water management, or the introduction of water-saving mechanisms such as direct wet seeding is favouring the development of high populations of *M. graminicola*, drastically raising its damage levels. Also, *R. similis* occurs only below ~1400 m altitude in the

East African Highlands where it is a principal pest of banana and plantain, a regional key starch staple for over 20 million people. A small raise in temperature would result in *R. similis*, which is cold-sensitive, infecting millions more bananas grown at higher altitudes (De Waele and Elsen, 2007, in Nicol *et al*., 2011).

1.5.4 Lag in nematode-genetic manipulation

Nematode control through genetic resistance is still insufficient although an extensive list of major annual and perennial crops carrying resistance to RKN, cyst and other PPN was recently reported (Molinari, 2011). Plant cultivars with high-standard agricultural traits of tomato, tobacco, cotton and groundnut resistant to *Meloidogyne* spp., of potato resistant to *Globodera* spp. and soybean resistant to *Heterodera* spp. are commercially available for growers (Starr and Roberts, 2004). While some crops benefit from resistance, many are susceptible; i.e. they lack identified resistant germplasm (McCarter, 2008). Furthermore, resistance-breaking through selection of virulent nematode populations (e.g. soy parasites) or selection for non-susceptible species (e.g. potato parasites) can take place, lessening the trait's value (Starr *et al*., 2002). Root-knot and cyst nematodes, and perhaps other species, have the capacity to develop new strains and races when cultivars resistant to these forms are planted too frequently (Sasser and Freckman, 1987). For example, five of nine populations of *Meloidogyne* spp. from Greece, identified as *M. javanica* (four populations) and *M. incognita* (one population) using either isozyme phenotypes or the sequence characterized amplified region-polymerase chain reaction (SCAR-PCR) technique, were virulent against the *Mi* resistance gene as assayed by pot experiments in controlled conditions. These populations could reproduce on tomato cultivars containing that gene (Tzortzakakis *et al*., 2005). Also, hypersensitive resistance (HR) to the common RKN species was clearly observed in infested roots of tetraploid potato clones that had been tested previously by UMR APBV-Potato team (Berthou *et al*., 2003; Kouassi *et al*., 2005), but such HR observations were not so distinct in the histopathological changes induced by Egyptian *M. incognita* populations probably because the Egyptian nematode population is more virulent (Eddaoudi *et al*., 1997) and/or other stressing environmental factors have accounted for such variations since some J_2 were active even after 10 days of inoculation (Abd-Elgawad *et al*., 2012). Moreover, despite the above-mentioned progress in molecular nematology, no transgenic approaches to resistance have reached commercialization and therefore PPN control lags behind transgenic control of insects, viruses and fungi. This lack of improved technology has been detrimental on several levels including nematology as a discipline and growers of susceptible crops. It has coincided with static-to-declining numbers of trained applied nematologists, particularly in the USA (McCarter, 2008). Race-non-specific (horizontal) resistance should be targeted as opposed to gene-for-gene recognition systems including HR (Keane, 2012). The genetic basis of resistance and virulence along with interactions leading to incompatible/compatible responses of the plants to nematodes and the role of some important hormones in plants and genetic variability of nematode populations were recently reviewed (Molinari, 2012).

1.5.5 Quarantine problems

Quarantine and certification programmes are important in limiting the spread of PPN but the programmes cause indirect costs in addition to direct loss. For example, *M. chitwoodi* and *M. fallax* are increasingly regulated as they can be spread through seed potatoes and potato tubers infested by PCN from Europe. As is widely accepted, quarantine cannot be feasibly carried out to all units of transferable plants, pots, soil and cuttings. Comprehensive precautions should be implemented for complete protection against a species as yet not found in a country (Salama and Abd-Elgawad, 2003). Complete certainty of pest absence can be assured only if every unit in the lot is inspected. Unfortunately, testing every unit is sometimes neither economical nor practical.

Therefore it is usually necessary to sample a portion of the units in the lot, and to accept or reject the entire lot based on the results. The inspector must assume some risk and set limits defining freedom from infestation, e.g. less than 1% of units infested, or less than five units infested, since it is a choice between objectives and available funds. Also, effect of sample size on the probability of detecting PPN species in a polyspecific nematode population and comparison of observed with expected rates of failure to detect this nematode by soil sampling in several locations were reported (Abd-Elgawad and McSorley, 2009).

Despite great efforts exerted on quarantine, striking examples of faulty quarantine regulations have led to the spontaneous introduction of nematode pests. *B. xylophilus*, associated with pine wilt disease but depending on bark beetles (*Monochamus* spp.) to spread from tree to tree, is native to North America and is thought to have been carried to Japan at the beginning of the 20th century on timber exports (Nicol *et al*., 2011). In Japan, it is causing massive mortality of native pine trees. In 1999, *B. xylophilus* was found in Europe for the first time, in Portugal. Quarantine nematologists were already researching the identity of this species in order to be able to distinguish it from the many species of *Bursaphelenchus* that inhabit wood. Unfortunately, there is a variation in characters between species in the *Bursaphelenchus* group, which makes morphological identification particularly difficult, so biomolecular tools, e.g. sequencing, species-specific primers and probes, are highly recommended (OEPP/EPPO, 2009a). Likewise, molecular and reliable protocols for quarantine species such as *G. rostochiensis* and *G. pallida* (OEPP/EPPO, 2009b), *M. chitwoodi*, *M. fallax* (OEPP/EPPO, 2009c) and *Xiphinema americanum* sensu lato (OEPP/EPPO, 2009d) were established while others are in progress. Generally, the cost of such protocols might vary from one country to another. For example, the double labeled TaqMan probe type might cost approximately US$200 in the USA, US$600 in Brazil and US$450 in Spain, from the same company, and also, it is possible to have different price/quantities and qualities depending on the company selected; it is conceivable that, hence, the rest of the reagents used for these protocols (master mix, plate, film, etc.) might change as well, although the function and use of these products are equivalent. Admittedly, in the countries where such technologies are currently under development and equipment not easily available, an extra implementation, with more costs, will be required for performing the same protocols. A comparison of these costs by countries or continents may give a very good idea of the potential and the limitations in including these molecular analyses. Moreover, such analyses require materials that sometimes are not considered in the initial budget, and at the end it significantly increases the final cost. It is important to highlight again that costs may also differ with other companies selected for the reagents or material, such as Applied Biosystem, Qiagen (http://www.qiagen.com) and IDT Technologies (http://eu.idtdna.com). Yet, at any rate, conducting such protocols will prevent the introduction and spread of quarantine species in new areas/states, thereby saving millions of dollars in annual crop losses (Handoo *et al*., 2013).

Holgado and Magnusson (2012) addressed the current status and perspectives of quarantine nematodes in the light of European legislation. They stressed the need to have actualized statistical data to provide information on problems emanating from particular sources, areas or suppliers, to determine priorities for targeting inspections and monitoring consignments with high risk to the agricultural industry in their countries and to perform a Pest Risk Analysis (PRA). Recent organic farming and the use of integrated pest management programmes, combined with the loss of many chemical products for PPN control, means that another future challenge is to develop a better understanding of the biology of PPN, so that as many cultural measures as possible can be used to suppress them at their source; e.g. increasing the interval between susceptible crops to reduce PPN multiplication rate. The growing desire to use plant waste for composting presents an additional risk unless appropriate measures are taken to secure complete sanitation. A good cooperation between the regulatory agencies, crop consultants, farmers and

growers is essential for success in all kinds of phytosanitary programmes. However, the increasingly declining skills in classical identification and diagnosis in nematology is evident. At the same time there are increasing demands to formalize quality procedures in laboratories, leading to the production of identification protocols that provide guidance for international agreement. The lack of resources for competence-building, maintenance and expansion of nematode collections will threaten the basis of identification of regulated nematodes. Hence, molecular techniques are especially important where morphological identification is particularly difficult or where only immature specimens have been intercepted. Today, diagnostic protocols are only developed for a restricted range of species, thus still necessitating a preliminary, provisional identification by a morphological specialist. Moreover, distinguishing races is a major challenge for inclusion of races on a list of regulated pests and further research is needed to develop rapid and specific methods for their distinction (Singh *et al*., 2013).

The lack of trained nematologists will lead to the spread of quarantine PPN such as *R. similis*, *Pratylenchus coffeae*, *P. goodeyi*, *M. chitwoodi*, *M. graminicola*, *M. mayaguensis*, *M. floridensis*, *G. pallida*, *H. glycines*, *D. dipsaci* and *B. cocophilus*, to mention but a few.

The constant increase in the transportation of food as dried seed or fresh produce ensures future spread of nematodes and thus underscores the need for trained nematologists in quarantine. The utilization of distribution maps to track serious PPN and to make decisions for new quarantine species gives support to the future need for a geophytonematological method to monitor the distribution of new and significant species for quarantine (Luc *et al*., 2005a).

Despite great advances in the use of molecular methods for the identification of diseases, their development as quarantine tools for phytonematode identification has been relatively slow especially in developing countries. According to the already existing or endemic PPN in a country, the quarantine listing of PPN species varies between countries. PPN species well known for their damaging impacts including for example *A. tritici*, *A. besseyi*, *A. fragariae*, *B. xylophilus*, *B. cocophilus*, *D. destructor*, *D. dipsaci*, *G. pallida*, *G. rostochiensis*, *H. glycines*, *H. schachtii*, *N. abberans*, *R. similis*, *X. americanum* and *X. index* are the most widely regulated (Singh *et al*., 2013). There are some other species that are damaging but not regulated in many countries often because they are usually widespread. However, few countries regulate specific races, e.g. *M. incognita*, *M. javanica*, *M. arenaria*, that are not present in their region. Race distinction is a major challenge for inclusion in the list of regulated pests and there is a need of further research to develop rapid and specific methods for the distinction of races. European quarantine nematodes include *M. chitwoodi*, *M. fallax*, *G. rostochiensis*, *G. pallida*, *H. glycines* (only for EPPO), *Hirschmanniella* spp. (except *H. gracilis* and *H. loofi*), *N. aberrans*, *R. similis*, *D. dipsaci*, *D. destructor*, *A. besseyi*, *B. xylophilus*, *Longidorus diadecturus*, *X. americanum* s.l. (non European), *X. americanum* s.s., *X. bricolense*, *X. californicum*, *X. rivesi* (http://www.q-bank.eu/Nematodes/DefaultInfo.aspx?Page=InfoQuarantine). Of these species listed in EU legislation, reliable protocols have only been developed for *B. xylophilus*, *G. pallida*, *G. rostochiensis*, *M. chitwoodi* and *M. fallax* (Holgado and Magnusson, 2012). Even as molecular tools can be used by personnel with no nematological skills, possible requirements in phytosanitary legislation to produce rapid morphological evidence will be difficult in the light of the declining competence in classical morphology among nematologists. Not recognizing the possible limitations in existing molecular techniques could in some cases result in over- or under-regulation of pest organisms. At present, the protocols for regulated species do not distinguish unregulated or native species of the genera that occur in the countries where interceptions or outbreaks may occur. For example, protocols for PCN do not include *Globodera achilleae*, a cyst nematode occurring in several European countries. PCN cysts will register as negative with such methods if eggs are absent, thus perhaps giving a false impression of the situation with regard to the status of the pest in a particular consignment. Therefore, the role of experienced diagnosticians and taxonomists in PPN identification remains a vital one.

1.5.6 Confusion in identification of some plant parasitic nematode species and races

The names of regulated PPN need to be as firmly established as possible. This requires awareness of the fact that some species might be subject to many taxonomic changes, and that there may exist many synonyms in the legislation of some countries; this needs to be recognized to avoid confusion and allow for the correct phytosanitary action to be taken. Examples of this controversy are *Radopholus citrophilus* and *R. similis*, which are both listed in European legislation. *R. similis* was thought to consist of different pathotypes but Huettel *et al*. (1984) concluded that the banana race and the citrus race were two distinct species; the name *R. similis* was restricted to the banana race and the citrus race was described as *R. citrophilus*. Later, Kaplan *et al*. (1997) synonymized *R. citrophilus* with *R. similis*; Valette *et al*. (1998) proposed *R. citrophilus* as a junior synonym of *R. similis*, although Siddiqi (2000) proposed it as a subspecies of *R. similis* and Elbadri *et al*. (2002), using molecular techniques, demonstrated marked intraspecific variation in various isolates of *R. similis*. This continuing taxonomic uncertainty has caused confusion for quarantine officers and specialists involved in PRA work, due to the uncertainty on the actual host lists of *R. similis* (Holgado and Magnusson, 2012). More recently, *M. enterolobii* and *M. mayaguensis* were considered separate species, but their recent synonymization meant that when distribution, host range and other information published under both names were consolidated, the apparent risk increased substantially. Therefore, while assessing phytosanitary importance, information published under species synonyms needs to be considered (Singh *et al*., 2013). On the other hand, splitting of a species (e.g. the new species *Ditylenchus gigas* and *D. weischeri*, previously considered as part of *D. dipsaci* species complex (Chizhov *et al*., 2010; Vovlas *et al*., 2011)) demonstrated that the distributions and phytosanitary importance of closely related or cryptic species and races could be assessed more precisely with more specific identification methods. Admittedly, uncertainties for the validity of nematode species will cause practical problems related to quarantine measures and nematode management as well as highlight the importance of research into taxonomy and specific identification methods.

1.5.7 Discrepancy in nematode technological progress among different countries

Addressing recent nematological issues will pose additional challenges to tropical and subtropical nematology since there has been discrepancy in nematode technological progress between the Northern and the Southern countries (Luc *et al*., 2005a). Moreover, the growing application of molecular diagnostics may widen the knowledge gap among nematologists especially between those in developed versus developing countries. Investigating the interactions between nematodes and other pathogens in disease complexes provides exceptional opportunities for multidisciplinary research with scientists from other disciplines but remains fairly underexploited, especially in developing countries. Contrary to developed countries, difficulties in recognizing emerging nematode threats in developing countries prevent the timely implementation of management strategies, thus increasing yield losses. For example, in the USA, the immediate implementation of a federal quarantine on the pale cyst nematode, *G. pallida*, as a serious pest helped prevent the spread of this species in the USA, thereby saving millions of dollars in annual crop losses (Handoo *et al*., 2013). This is especially important since over-population occurs predominantly in developing, mostly tropical and subtropical, countries where the majority of hungry people live. So, reducing yield losses caused by pathogens of tropical agricultural crops is one measure that can significantly contribute to increased food production due to vast arable lands there. The future for subtropical and tropical nematology will likely be long and full of complex and economically important problems especially with regards to subsistence agriculture (Luc *et al*., 2005a). Of utmost importance to nematology in the future will be access to centres with competence in systematics similar to those in developed countries.

1.5.8 Inaction or shift in plant parasitic nematode management

Sometimes even in the developed countries, forgotten nematode problems can reappear all of a sudden as rotation sequences are altered or new cultivars introduced, as has been seen with new outbreaks of PCN and sugarbeet stem nematode *D. dipsaci* (Luc *et al*., 2005a). A problem new to a particular country could arise through the introduction and subsequent spread of a known nematode parasite from another temperate country. Therefore, in the case of temperate countries, surveys are designed to determine the distribution of known nematodes causing known damage. Contrary to this, in the subtropical and tropical areas, new problems are being, and have yet to be, discovered involving new nematode species and even genera, or species not previously recorded as detrimental to a crop. Examples are the 'legume Voltaic chlorosis' of leguminous crops, discovered in Burkina Faso, associated with a new species, *Aphasmatylenchus straturatus*, and a genus not previously known to be a harmful parasite (Germani and Luc, 1982a, b); 'miti miti' disease of taro (*Colocasia esculenta*) in the Pacific caused by a new species, *Hirschmanniella miticausa* (Bridge *et al*., 1983); and, in the semiarid areas, the new cyst species *Heterodera ciceri* causing damage to chickpeas and lentils (Greco *et al*., 1984; Vovlas *et al*., 1985); *Meloidogyne mayaguensis* (Rammah and Hirschmann, 1988), now widespread on many crops; *Achlysiella*, a new genus and potentially damaging pest of sugarcane (Hunt *et al*., 1989); *Radopholus citri*, highly pathogenic on citrus in Indonesia (Machon and Bridge, 1996); *M. paranaensis* (Carneiro *et al*., 1996), now a devastating pest on coffee in Brazil; and *Radopholus duriophilus*, found widely distributed on durian in Vietnam associated with decline and death of trees in many durian nursery gardens (Nguyen *et al*., 2003; in Luc *et al*., 2005a). Yet, since nematodes are highly adaptable and capable of surviving in different environments and, so far, only a small percentage of the total species are known and as new species are discovered, they reveal previously unknown agricultural problems. For example, in Egypt, *A. besseyi* causing white tip leaf disease on rice was detected (Amin, 2001) and most recently aerial shoot galls on camphor trees induced by the plant parasitic nematode *Fergusobia* spp. and the dipterous insect *Fergusonina* spp. were reported in several Egyptian governorates during winter, spring, summer and autumn seasons (Kella and Bekhiet, 2011).

1.5.9 Lack of economically oriented plant-parasitic nematode research

We believe that more economically oriented research work should be followed. McCarter (2008) proved this case by figures. About half of the total (48%) loss attributed to PPN derives from only two crops, rice (Bridge *et al*., 2005) and maize (McDonald and Nicol, 2005), which prevail due to their overall domination in global agriculture. Twenty-eight per cent of the total loss results from rice in China and maize in the USA alone. Although the figures of nematode losses in rice and maize are staggering, few molecular nematologists focus their research on these crops and the associated nematode pathogens. Many PPN parasitize rice, but studies of their related diseases and yield loss in rice are limited (Padgham *et al*., 2004; Bridge *et al*., 2005), and international agricultural research centres have employed a minimal number of nematologists (Luc *et al*., 2005a). Despite cases of substantial yield loss documented in certain states, such as PPN damage of maize hectarage in Nebraska (McCarter, 2008), little agricultural extension has been supplied to US maize growers or breeders to make the case for a focus on PPN control as a yield-boosting strategy (Koenning *et al*., 1999). Growing market demand for US maize (e.g. ethanol production) may draw more attention for better yield traits like PPN resistance, which are underestimated by industry.

1.6 Resources and Facilities Devoted to Nematology versus Limitations

Resources and facilities devoted to nematology differ from one country to another. Generally, developed countries offer more and better

resources to nematology than under-developed countries and those revolving around the developing level. Sasser (1988) estimated resources devoted to nematology in terms of personnel and financial support based on the above-mentioned questionnaire. He found that although many nematologists have adequate financial support for their programmes, 65% indicated that they were not satisfied with the support received when compared to other disciplines; 27% were satisfied and 8% did not respond to this question. In spite of problems with support, a large number of professional societies exist, and many publish their own journals at regular intervals. Currently, such societies and federations include: Afro-Asian Society of Nematologists (AASN), Australian Association of Nematologists (AAN), Brazilian Nematological Society (Sociedade Brasileira de Nematologia) (SBN), Chinese Society of Plant Nematologists (CSPN), Egyptian Society of Agricultural Nematology (ESAN), European Society of Nematologists (ESN), International Federation of Nematology Societies (IFNS), Italian Society of Nematologists (Societa Italiana di Nematologia) (SIN), Japanese Nematological Society (JNS), Nematological Society of India (NSI), Nematological Society of Southern Africa (Nematologiese Vereniging van Suidelike Afrika) (NSSA), Organization of Nematologists of Tropical America (ONTA), Pakistan Society of Nematologists (PSN), Russian Society of Nematologists (RSN) and Society of Nematologists (SON). Nematology periodicals (publishing societies or organizations shown in brackets) include: *Asian Journal of Nematology* (AJN), *International Journal of Nematology* (ASSN), *Nematologia Brasileira* (SBN), *Journal of Nematology* (SON), *Nematology Newsletter* (SON), *The Egyptian Journal of Agronematology* (ESAN), *Egyptian Society of Agricultural Nematology Newsletter* (ESAN), *Nematology News* (ESN), *Nematologia Mediterranea* (Istituto di Nematologia Agraria of the CNR), *Japanese Journal of Nematology* (JSN), *Indian Journal of Nematology* (NSI), *African Plant Protection* (NSSA), *Nematropica* (ONTA), *Organization of Nematologists of Tropical America Newsletter* (ONTA), *Pakistan Journal of Nematology* (PSN), *Pakistan Society of Nematologists Newsletter* (PSN), *Russian Journal of Nematology* (RSN) and *Nematology* (Brill Academic Publishers).

In the 1980s, 64% of the respondents (Sasser, 1988) expressed dissatisfaction with the image of nematology at their institutions. Most of the suggestions to improve this image centred on increased public recognition of nematode damage, development of effective management options, better research programmes and greater funding. The hiring of more nematologists and support staff was deemed critical to image improvement. Many respondents indicated that they felt constrained in their work by lack of facilities and supplies, administrative support, funds, technical assistance and communication with other nematologists. The continuation of such limitations, in the under-developed countries and those revolving around the same limit, has currently hindered them to keep pace with developed countries. For example, in nematode taxonomy, nematode collections still remain as individual personal collections that are not well organized or maintained; unfortunately, many valuable specimens are subject to deterioration or are misplaced and collection records might be lost. On the contrary, such collections in the USA can serve as an official permanent repository that is well organized and contains detailed records of all specimens collected from different countries. Also, a preventative maintenance programme was established so that aging slides can be periodically remounted as needed, and the process of salvaging material from the early USDA nematologists remains an ongoing effort. Over time the collection has steadily expanded and now includes over 45,000 slides and vials, most of which are stored in fireproof safes (Handoo *et al*., 2013). All records of the US Department of Agriculture Nematode Collection (USDANC) are now digitized, stored and maintained in a computer database. Currently, the database is configured so that any new records are automatically uploaded to a USDA server and are then instantly searchable and available to the public at http://nt.ars-grin.gov/nematodes/search.cfm. This instant upload configuration represents a great improvement over previous upload methods and greatly improves the timeliness that information can be made available to the public. The database currently contains over 39,000 entries with essential data on

nematode hosts and distribution recorded for each species. While the majority of specimens are plant-parasitic, the collection also includes many free-living, insect-parasitic, marine and freshwater nematodes. Moreover, new multimedia/multi-platform nematological presentations are easily and freely available; e.g. both 'Introduction to Nematodes' and 'History of the Society of Nematologists' can be downloaded from several websites such as http://www.nematologists.org. So, tools made available in recent decades (scanning electron microscope, gel electrophoresis, computers, polymerase chain reaction (PCR), real time qPCR, World Wide Web of online datasets, molecular kits for nematode identification, etc.) have allowed us to make rapid advances in taxonomy and biochemistry of nematodes as well as systems science and data management and there are many challenging and exciting areas of research that will yield high dividends. Methodology for evaluation of BCAs against PPN is rapidly developing, and support for this area of research is likely to increase in the years ahead. Financial support could develop modern techniques to study and analyse new nematode–host associations while nematode-tolerant and/or -resistant crop plants are being discovered each year with many new nematode species/races. As a result, more durable and sustainable resistance is being tracked. Rapid and reliable nematode identification is essential and therefore additional funds should be allocated for new quarantine techniques. Other PPN management options such as crop rotation, nematicides, grafting and soil solarization are being improved and/or upgraded. It is also encouraging to note that many growers and agricultural companies can surf the internet to gain knowledge about PPN as well as to learn more about their management.

On the other hand, a big disadvantage of concentrating on the taxonomic aspect in developing countries is that the surveys are designed to collect nematodes and not to determine the problems caused by nematodes. This is often the only possible means of establishing new nematology laboratories with limited staff and financial means. The danger is that such laboratories, probably due to limited funds, can limit their activities to systematics and so become production lines, for new species and genera, to the exclusion of determining the importance of the nematode being described. So, the practical problems of determining nematode pathogenicity in the tropics can often be far more difficult than in temperate countries (Noe and Sikora, 1990). Difficulties arising from maintaining controlled conditions in glasshouses with different devices because of the excessive heat can be a daunting and expensive task (Luc *et al*., 2005a). Knowing which nematode genera and species occur is the necessary first step, but establishing the pathogenicity of the nematodes involved in subtropical and tropical agriculture has to be made a main priority (Luc *et al*., 2005a). Many nematodes are now recognized as serious or potentially serious pests of tropical crops but information on the actual yield losses caused by the nematodes in different situations and on different crops is still sadly lacking for a large proportion of these nematodes. This knowledge is essential to provide agricultural scientists, extension officers and administrators with the knowledge necessary to recommend practical and economic means for PPN control in the face of all the other constraints on crop production. Admittedly, numerous textbooks show the extent of damage that can be caused by nematodes, which is recognized by the nematologists concerned but generally not by other agriculturists. Crop damage by PPN invariably remains hidden by the many other limiting factors in subtropical and tropical agriculture, especially the existence of multiple biotic and abiotic stress factors operating simultaneously on the crop. In fact, from the early 1930s until recently, the bulk of researchers studying nematodes have been plant pathologists in training on a worldwide basis (Van Gundy, 1987). As a result, many researchers have studied nematodes as part of their training. Consequently, PPN research depended heavily on answering plant pathological and agro-economical questions for the last decades. However, even with these PPN diagnostic and advisory services, farmers may experience heavy losses because of the diversity of PPN genera in fields and because new races often emerge after repeated use of resistant cultivars. Thus the number of available

resistant cultivars diminishes in relation to their actual usefulness in the suppression of damage. The effective use of available resistant cultivars should be maximized by their integration with other PPN management practices.

Funding is critical to nematology. Sasser and Freckman (1987) estimated that less than 0.2% of the crop value lost to nematodes worldwide is used to fund nematological research to combat these losses, which probably exceed US$100 billion annually. This percentage might still be a valid guess now despite the increase in spending on different aspects of nematology; more losses caused by PPN are also recognized. Perhaps, if we can convey the imbalance between crop losses and expenditures to combat these losses to appropriate government officials and to funding agencies worldwide, then more resources may be allocated for nematode research, teaching and extension. Increased recognition of PPN problems and their due losses may serve this trend. Because the highest crop losses due to nematodes occur in developing countries, the dollar-value losses are greater there than in developed countries. The percentage funding for nematological research in the tropics and developing countries is considerably less than it is in most of the temperate and developed countries. With few exceptions, the efforts and resources directed towards research on PPN within the International Agricultural Research Centers (IARCs) have been and remain much less than even a conservative assessment of their significance as crop pests would merit (Sharma *et al*., 1997). Examination of the Senior Scientific Staff in the IARCs over a 20-year period showed that numbers of nematologists remained unchanged at a bare minimum even though there was an increase in other disciplines.

Due to the present trend of down-sizing in all fields of agricultural research, and thereby the loss of many diagnostic laboratories, qualified taxonomic identification will be a problem in many countries especially in quarantine where decisions on nematodes detected in samples, in particular species and race designations, need to be made almost spontaneously (Luc *et al*., 2005a). Now, it may be necessary to develop 'virtual centres of excellence' in diagnostics for use by nematologists working in the tropics/less-developed countries to support nematology in the field of species identification. Also, within the same context of comparing nematology in 'temperate' with 'subtropical and tropical' regions, it is appropriate to raise the obvious questions of whether there are still fundamental differences (Noe and Sikora, 1990; Luc *et al*., 2005a) or whether they differ only in degrees because of the different species of nematodes and types of crop present. Climate definitely affects PPN distribution on a geographical scale since most nematode life processes have thermic optima that determine the ideal geographic ranges of nematodes. Presumably, there are southern and northern hemisphere bands of appropriate temperatures for each nematode species that would be contiguous and would meet at the equator for true tropical species.

Recently, the number of scientific research papers published annually has decreased in the above-mentioned nematological periodicals, despite their high scientific level. Consequently, some of these periodicals, e.g. *Nematropica* and *Journal of Nematology*, have abandoned page charge fees to encourage nematologists to pay only journal subscriptions and publish free of page charges. A few years ago, two journals (*Fundamental and Applied Nematology* as well as *Nematologica*) were merged together into one, which is now published as *Nematology*. Furthermore, some of these periodicals are threatening to stop publishing due mainly to limited funds; the world economy has not recovered yet from the 2008 global financial crisis. Another high standard journal, *Nematologia Mediterranea*, is not published anymore. The main reasons are: (i) lack of funding; (ii) the time that the editors have to devote to it is not recognized by others; and (iii) nematology journals have very low impact factors (Nicola Greco, CNR at Bari, Italy, 2013, personal communication).

Due to economic constraints, research in nematode management in the tropics often focuses on low-input methods involving crop rotations, multicropping, adjustment of planting and harvest dates, use of various soil amendments and mulches, trap and antagonistic crops, fallow, flooding, etc. (Sikora *et al*., 2005).

Agricultural scientists working in the tropics and subtropics are emphasizing these forms of control strategies. This shows increased awareness of the need for nematode management systems that rely less on the use of nematicides. However, new management tools have been developed that have widened the integrated pest management toolbox, including solarization, biological control, trap cropping, resistant rootstocks, biofumigation, molecular kits for root-knot identification, remote sensing and precision farming, nematicide formulation and application technology. So, none of the PPN problems are insurmountable with the appropriate trials, expertise and support. With the progress of time, nematologists have accumulated a great deal of recent knowledge on the significance of nematodes as plant parasites and the successes in their management. The statement can be justified by citing an example that a literature search of CABI abstracts for plant parasitic nematodes and vegetables yielded over 2800 citations for the period between 1990 and 2003 (Luc *et al*., 2005a). However, as far as developing countries are concerned, nematology is underfunded and there is a lack of nematologists to work on the problems.

1.7 Economic Framework of Phytonematodes

The current literature on references and illustrations of the above-mentioned phytonematodes is voluminous and impressive in the field of their biology, diseases and control (Evans *et al*., 1993; Luc *et al*., 2005a; Jones *et al*., 2013), and is not updated and relatively incomplete in that of economic analysis and cost-related phytonematode issues as can be seen from Khan (1972), Noling (1987), Sasser and Freckman (1987) and McCarter (2008). We intend here to throw light on the main framework and economic foundation in nematology.

The economic consequences of crop losses due to PPN may be categorized into different levels; the farm producer, consumer and society/community. The farm producer suffers the loss of revenue or the increased cost of production because of the reduction in crop yield, the loss in quality of the marketable produce and/or costs of nematicide and its application. In addition, there is the factor of uncertainty which the farmer must face (Khan, 1972). Needless to say, if the additional costs of nematicides cannot save much additional value or returns, these controls cannot be regarded as profitable. There is also the loss to the consumer, household and industry, either through the price increase or through the deterioration in quality of the consumable product or a combination of these two. Some health hazards might be implicit on consuming produce treated with banned nematicide (Abd-Elgawad, 2008). Moreover, there is the loss to society, of which the producer and consumer are a part, in the form of wasted resources which have direct and implicit costs. Losses to society may appear in other forms such as the payment of relief and subsidies to the farmers and in some instances to the consumers. The national resources so diverted could have been utilized for some other purposes. This often leads to the non-optimum use of a society's economic resources. In countries that are economically weak and agriculturally backward, these losses could be critical. Crop losses due to PPN in these countries may defeat the efforts to increase production and efficiency. There may be yet another type of loss that adversely affects all these levels if the nematode disease is of a persistent character, in the sense that it leaves residual effects in soil and/or plant residues, which will affect farm planning for several seasons or even years. This usually happens with uncontrolled PPN-infested farms and could force the farmer to re-allocate his resources. Since PPN are often soil-dwelling pathogens, selecting a profitable management option is necessary to maintain their population densities below the damaging level or at most the economic threshold level. Finally, there is the loss sustained by the world community, especially in those parts of the world that are struggling against food and raw material shortages, population growth and slow development. These losses are not confined to food crops. They affect also the cash crops which form a major source of revenue to the farmers and the industry (Khan, 1972).

The relative importance of each specific crop yield also plays a significant economic

role. Usually, the initial effect is that there is a decrease in marketable supply, but if it occurs on farm(s) whose producer is not an influential supplier, it is most likely to reduce the total revenue of this producer because he cannot determine the market price of the product by himself. On the contrary, if the disease affects the majority of the suppliers in a market, the economic scene changes. As for the price and revenue effects on the farmers, much will depend on the nature of demand, or the so-called elasticity, for the product so affected. Elasticity refers to the ratio of the percentage change in quantity to the percentage change in price (Khan, 1972). Staple crops such as wheat, barley and rice have a relatively 'less elastic' demand than fruits, e.g. apples, grape and citrus. The conditions of demand for such staples and non-staples may differ since they occupy different positions in the consumer's budget and preference scale. For example, apples are likely to have more close substitutes than has wheat. Assume that the supply conditions for these two are similar; the decrease in the supply of the two crops caused by PPN will have different results. Buyers of wheat or staple, contrary to apples or non-staple, foods are less responsive in terms of the change in quantity than to a change in the price. So, owing to nematode diseases on both wheat and apples, the supply is reduced: less of these two products are offered. Assume that the demand conditions remain unchanged. The result is obvious: that the prices of wheat and apples will increase and the quantities bought and sold will decrease. Yet, the changes in the price and quantity of wheat are likely to increase the total producer's revenue but will reduce it in the case of apples. While the price and quantity changes are similar in both instances, in the case of wheat the farmers stand to gain and in the apples they may lose due to the different elasticity. In reality, it is not as simple as that. The reduction in supply may also mean that farm resources have not been used optimally or at the lowest cost per unit of output. First, in instances where additional revenue has accrued through price increase, it may not equal the cost that waste in resources implies. Second, the wasted resources could have been used optimally had they been put to some other crops. Finally, to a single farmer, who may be the only one affected adversely by crop losses due to PPN, the total loss may even be greater. On the other hand, if we assume that the farmers properly use nematicide to save the yield and the quality of wheat and apples to be marketed, the yield will increase. In the case of wheat, the price will likely decrease resulting in a loss of total revenue to the farmers, while for apples, this may increase the total revenue. So, it is not always true that disease control will increase the farmer's total revenue. Further, the costs of control may be excessive, despite the fact that the farmers save their revenue. Admittedly, combining the biological and economic aspects of nematode diseases and their control should improve the existing methods of evaluation of losses and benefit the parties for whom the research work is done. If the cost–benefit ratios are not favourable, a comparison may have to be made between the situation with no control and the alternative use of farm resources. In some instances, it may be found that the costs and revenues will be favourably affected by shifting the resources to some other use (Khan, 1972). Hence, the total farm picture should be analysed to achieve the economic principle; i.e. best maximization of ends, optimum minimization of means, or both together.

1.8 Conclusions and Future Prospects

In the foregone literature, current losses due to PPNs have been estimated up to the tune of US$358.24 billion annually on a worldwide basis, which is undoubtedly a serious threat to the world economy. The economic importance of a PPN is judged by its parasitic or pathogenic potential, geographical distribution and value of the crop. Several factors are responsible for yield loss but the yield loss may even occur without any visible symptoms (Bos and Parlevliet, 1995). One main drawback in this context is that the damage caused by PPNs is generally related to the value and importance of the crop concerned to a particular region or

country and that is why we have much information on some specific species of nematodes like *G. rostochiensis*, *G. pallida*, *H. glycines* and *Meloidogyne* in temperate zones but not much on the same nematodes in subsistence agro-ecosystems (Manzanilla-Lopéz *et al.*, 2004). There are few diseases caused by root-knot and cyst nematodes which are attracting attention of the researchers all over the world and allocation of research funds are generally restricted to the major PPN species attacking high-value crops. This is one of the reasons that limit/restrict our knowledge of other nematode diseases on many crops and their effect on the agricultural economy.

In recent years, research has been done in different institutes, research stations and laboratories all over the world but still our knowledge about nematode problems is meager, particularly in developing countries where there is a lack of trained nematologists. For any successful treatment, the starting point should be the correct diagnosis of a disease and this can be achieved with a well-trained professional in the field. There are several reasons that lead to confusion in recognizing nematode infection on plants. One among them is the earlier aboveground symptoms on a plant which is very similar to that caused by fungi or bacteria or nutritional disorders. It is rare that nematodes directly kill any plant, instead they draw their nutrition from plant hosts, which in turn debilitates the plant to some extent. Losses that result from nematode attack may not necessarily be as a consequence of direct cell death, necrosis or 'diseased' tissue but may derive from other more insidious aspects, such as interference with the root system that reduces their efficiency in water and nutrient uptake. These causes are generally misdiagnosed. In most cases, the farmers are unaware of nematodes as their enemies or, if aware, feel helpless as they do not know any practical and economic means of combating them even after four decades of organized basic and applied nematological research in the world. The reason needs investigation and reappraisal of our research priorities. Perhaps the research approach has remained alienated from the local conditions, or endeavours and recommendations of research workers have not reached the farmer or both may be the cause.

Nematologists are now aiming to manage crop losses to obtain maximum profit with the least disturbance of the ecological balance. In the last two decades while combating against phytonematodes, particular emphasis has been laid upon environmental health hazards, less dependence on chemical nematicides, and the search for an alternative safe method of nematode management, but in the decades to come the rapid expanding global human population will have to complete among themselves for food and fibre on the same fragile agro-ecosystem. Under those circumstances the adverse role of PPNs, which has certainly posed a threat to world crop production, needs to be checked. This is a challenging task for present and future nematologists.

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2 Significance of Biocontrol Agents of Phytonematodes

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2.1 Introduction

Plant parasitic nematodes (PPNs) pose a major constraint on world agriculture resulting in significant yield losses especially in the developing countries where suitable and effective control measures are unavailable (Webster, 1987; Nicol *et al*., 2011). It is estimated that PPNs impose 8.8% and 14.6% annual losses to developed and developing countries, respectively (Nicol *et al*., 2011), which are more or less equal to US$157 billion (Abad *et al*., 2008; Escudero and Lopez-Llorca, 2012), however the exact loss estimation of PPNs is too difficult (Schomaker and Been, 2006). Their microscopic size, underground existence and non-specific symptoms make their presence often undetected; therefore, the diagnosis of nematode problems are frequently confused with nutritional deficiencies or other soil factors (Perry and Moens, 2011).

The main option for managing PPNs has been chemical nematicides, but their application is now being reappraised according to environmental hazards, mankind health, availability and expense (Davies and Spiegel, 2011a; Moosavi and Zare, 2012). The effective chemical nematicides are not affordable by many farmers and have mostly been taken off the market due to health risks posed to the users and environment (Thomason, 1987; Quesada-Moraga *et al*., 2014). Frequent application of other available nematicides can result in emerging resistant nematode races, which in turn will make their control more difficult (Narayanasamy, 2013). These problems along with raising social awareness on health concerns and ecological hazards of chemical pesticides have caused an urge to search for other safer alternative methods. Among the available control options, biological control is gaining widespread attention because it promotes sustainable agriculture and environmental protection.

In wild natural environments, PPNs are just one component of the numerous organisms that live in soil and their population densities are subjected to a constant series of checks and balances that stabilize their populations between certain upper and lower limits. 'Biological control' is normally used to describe the effect of soil biota in decreasing the nematode populations to lower average levels than would occur in their absence (Stirling, 2011).

Nematode populations are influenced by many soil organisms that include the microfauna (e.g. fungi, bacteria, viruses and protozoa), mesofauna (e.g. rotifers, nematodes, tardigrades, collembolans, mites and enchytraeids) and macrofauna (e.g. earthworms, termites and

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millipedes) (van der Putten *et al*., 2006; Costa *et al*., 2011; Stirling, 2011). However, the success in implementation of these biocontrol agents (BCAs) greatly depends on our understanding on soil ecology. The biological control potential of fungi and bacteria against PPNs has been well demonstrated. However, predatory soil nematodes, mites and viruses have also been found to have biocontrol activity against PPNs.

The focus of this chapter lies upon the significance of different BCAs in the management of PPNs with particular emphasis on nematophagous fungi and bacteria. Recent developments on this issue have also been reviewed to commercialize these beneficial microorganisms and include them in an integrated nematode management (INM) programme.

2.2 Nematophagous and Endophytic Fungi

Nematode antagonistic fungi have an important effect in the regulation of PPN populations and various aspects of their biological control against nematodes have extensively been reviewed by numerous workers (Stirling, 1991; Siddiqui and Mahmood, 1996; Kerry, 2000; Walia and Vats, 2000; Kerry and Hominick, 2002; Chen and Dickson; 2004; Jansson and Lopez-Llorca, 2004; Viaene *et al*., 2006; Lopez-Llorca *et al*., 2008; Hallmann *et al*., 2009; Timper, 2011; Moosavi and Zare, 2012; Pendse *et al*., 2013). All nematode-parasitic and antagonistic fungi could be classified into two large groups: nematophagous fungi and endophytic fungi. Nematophagous fungi include egg- and female-parasitic fungi, nematode-trapping fungi, endoparasitic fungi and toxin-producing fungi. The endophytic group consists of mycorrhizal fungi, *Neotyphodium* endophytes and *Fusarium* endophytes (Porras- Alfaro and Bayman, 2011; Moosavi and Zare, 2012). Interestingly, recent studies illustrate that a considerable number of fungal pathogens of invertebrates, including nematophagous fungi, have an endophytic phase in their life cycles (Quesada-Moraga *et al*., 2014).

Several species of nematophagous fungi are obligate parasites, while the majority of them are facultative (Lopez-Llorca *et al*., 2008) and external and/or internal signals may stimulate the switch between saprotrophic to parasitic phase (Macia-Vicente *et al*., 2011; Yang *et al*., 2011a; Ananko and Teplyakova, 2013). The obligate parasites typically infect nematodes by their spores, which may be ingested or may adhere to the nematode cuticle, while facultative ones usually produce trap structures, adhesive spores or appressoria (Moosavi and Zare, 2012). Nematophagous fungi live in nearly all types of soil (Dackman *et al*., 1992), however they have less commonly been found in aquatic environments (Hao *et al*., 2005). They can consume either one or more stages of living nematodes (eggs, juveniles, vermiform adults and sedentary females) by various mechanisms (Jansson and Lopez-Llorca, 2004).

Endophytic fungi colonize the interior plant tissues without causing any lesions or other disease symptoms. These fungi are considered mutualistic if they improve the plant growth and health (Schulz and Boyle, 2006; Sikora *et al*., 2008). Dependence of these organisms on plants varies from obligate endophytes, which totally rely on living plant cells, to facultative endophytes, which can exploit both living and dead organic matters (Hallmann and Sikora, 2011). Fungal endophytic association has been reported for nearly all plant species from the Arctic to the tropics (Kuldau and Yates, 2000; Aly *et al*., 2011; Hallmann and Sikora, 2011) with few exceptions (Lambert and Casagrande, 2006). It has been demonstrated that endophytic associations of fungi in plant roots are mainly systemic with inter- and/or intracellular establishment (Macia-Vicente *et al*., 2011). It appears that nematophagous and entomopathogenic fungi have derived from plant pathogenic fungi to escape competition from other soil biota by more specialization in alternative hosts (Barron, 1992; Bidochka *et al*., 1999). Consequent to this evolutionary progress, they obtained the abilities of altering hosts among nematodes (or insects), endophytism and mycoparasitism (Lopez-Llorca and Jansson, 2006; Moosavi *et al*., 2011).

Endophytic fungi can be divided into mycorrhizal fungi, *Neotyphodium* endophytes and *Fusarium* endophytes (Porras-Alfaro and Bayman, 2011). Mycorrhizal fungi are the best known endophytes, forming a symbiotic

association with nearly 80% of all plant species (Wang and Qiu, 2006). Arbuscular mycorrhizal (AM) fungi are the most common mycorrhiza with obligate symbiotic association with their plant hosts (Veresoglou and Rillig, 2012). Ectomycorrhiza, ericoid mycorrhiza, orchid mycorrhiza and mycoheterotrophic mycorrhiza are other types of mycorrhizae (Smith and Read, 2008). AM fungi improve the growth of nematode-infected plants and could decrease nematode infestations, while very little is known about the impact of other forms of mycorrhizae on PPNs (Villenave and Duponnois, 2002; Jung *et al*., 2012). Presence of fungal species with endophytic growth in all fungal divisions proposes the independent evolution for endophytic colonization on many occasions (Hallmann and Sikora, 2011). Species of *Neotyphodium* (former *Acremonium*) are obligate biotrophs with intercellular growth, which commonly colonize the cool-season grasses. *Neotyphodium* endophytes establish a symbiotic association with plants whereas fungus supports the plant against abiotic and biotic stresses and the plant provides protection and nutrition to the fungus (Timper *et al*., 2005; Sullivan *et al*., 2007). This endophytic fungus could enhance the resistance level of its grass partner to some but not all PPNs (Hallmann and Sikora, 2011).

Members of the genus *Fusarium*, in particular *F. oxysporum*, are the most ubiquitous endophytic fungi that were isolated from nearly all examined plant species (Kuldau and Yates, 2000; Bacon and Yates, 2006; Macia-Vicente *et al*., 2008). Suppression potential towards PPNs has been reported for many non-pathogenic *F. oxysporum* strains, as well as other non-pathogenic *Fusarium* species (Hallmann and Sikora, 2011).

2.2.1 Mode of parasitism

Nematode-trapping fungi develop different structures such as adhesive hyphae, adhesive branches, adhesive nets, adhesive knobs, non-constricting rings, constricting rings and stephanocysts for entrapping nematodes (Chen and Dickson, 2004; Liu *et al*., 2009; Moosavi and Zare, 2012). Most nematode-trapping fungi occupy the bulk soil and wait for contact with their prey. Attraction of nematodes toward the traps by producing secondary attractive compounds (Hallmann *et al*., 2009) or eavesdropping on chemical communication among their prey (Hsueh *et al*., 2013) increases their trapping opportunity. Secretion of proteolytic enzymes, antimicrobial and nematicidal compounds also enhances their aggressiveness (Moosavi and Zare, 2012).

Endoparasitic fungi usually infect vermiform nematodes by their spores (conidia or zoospores), which are ingested by the prey and germinate in its intestines, or stick tightly on its cuticle when it passes the fungus (Lopez-Llorca *et al*., 2008). Zygomycetous endoparasitic fungi produce motile zoospores that swim towards the nematode, attach to the cuticle and enter the host body via natural openings (Viaene *et al*., 2006). Fungal species with ingestible conidia usually parasitize bacteriophagous nematodes, as the broader mouth opening of these nematodes increase the possibility of swallowing conidia (Moosavi and Zare, 2012).

The infection pattern of egg- and female-parasitic fungi against cysts, saccate adult females and eggs are usually similar (Lopez-Llorca *et al*., 2008), however the egg- and cyst-parasitizing fungi are more numerous than those infecting females (Kerry and Hominick, 2002). The egg- and female-parasitic fungi normally use appressoria or zoospores to parasitize their hosts (Lopez-Llorca *et al*., 2008). The shell of nematode eggs consists of three distinct layers with protein and chitin as its main structural components (Perry, 2002) while nematode cuticle is a three-zoned structure consisting predominantly of collagen-like proteins along with other proteins, lipids and carbohydrates (Lee, 2002; Curtis *et al*., 2011). Egg- and female-parasitic fungi use both enzymatic and physical means for passing through their hosts' surfaces. They usually employ an appressorium, a specialized penetration peg or lateral branches of mycelium for penetrating into the eggshell or cuticle of the nematode (Lopez-Llorca *et al*., 2008) together with extracellular enzymes such as protease, chitinase and collagenase (Macia-Vicente *et al*., 2011). Secretion of extracellular enzymes is one of the most significant determinants of fungal virulence. Numerous studies have recently been carried out to identify and characterize these enzymes and to clone their genes (Liang

et al., 2010, 2011; Liu *et al*., 2011; Larriba *et al*., 2012; Braga *et al*., 2013; Soares *et al*., 2013; Yang *et al*., 2013). Gene manipulation of BCAs for over-expression of cuticle- and eggshell-degrading enzymes has been performed in order to increase nematicidal activity (Ahman *et al*., 2002; Yang *et al*., 2011b). Zoospore-producing species can infect the female nematode and impede cyst formation. They are also capable of producing resting spores that survive in soil when their host is absent (Moosavi and Zare, 2012).

As the name 'toxin-producing fungi' implies, they secrete nematicidal or nematostatic compounds that immobilize their prey prior to penetration through its cuticle (Lopez-Llorca *et al*., 2008). A number of compounds with antibiotic (nematicidal and antifungal) activities have been isolated from different groups of nematode-antagonistic fungi (Kerry and Hominick, 2002; Anke, 2011). It is estimated that about 40% of 50,000 known microbial metabolites are produced by fungi while approximately half of them possesses antibiotic activity (Berdy, 2005; Buckingham, 2008). Considering the fact that only 50,000 species (Anke, 2011) of 1.5 million fungal species estimated to exist (Hawksworth, 2001) have been screened so far, it seems that researchers may be able to discover many nematicides from fungi in the near future. A commercial *Myrothecium verurrucaria*-based product is now available with the name of DiTera®. This fungus is a filamentous ascomycete that produces nematicidic compounds (Wilson and Jackson, 2013).

Endophytic fungi can give some advantages to their host plants such as growth promotion, secretion of secondary metabolites against plant pathogens, reduction of palatability and stimulation of host's systemic resistance (Macia-Vicente *et al*., 2011; Mayerhofer *et al*., 2013). AM fungi are able to suppress nematode diseases by 44–57% (Veresoglou and Rillig, 2012). They may use one or a combination of several mechanisms against PPNs. There are contradictory reports on the possibility of competition for space between nematodes and AM fungi. Some researchers consider it probable in consequence of the fact that both PPNs and AM fungi are obligate organisms, depending upon intact root tissues for development (Hallmann and Sikora, 2011), but others have doubts (Hol and Cook, 2005). AM fungi assist the host plant better to tolerate nematode infection by improving its nutrient status (Azcón-Aguilar and Barea, 1996). Although these are evident benefits of an enhanced nutritional level for increasing plant tolerance to nematodes, it has been shown that the protective effect cannot only be attributed to higher nutritional status (Jung *et al*., 2012). Involvement of induced resistance against PPNs is another hypothesis which has both supporting and denying evidences (Hallmann and Sikora, 2011). Ectoparasitic nematodes cause more damage to AM fungi-associated plants than non-AM fungi-associated ones. Contradictorily, endoparasitic nematode damage is less for AM fungi-associated plants (Hol and Cook, 2005). Acaulosporaceae has been found the weakest family of AM fungi in suppression of PPNs (Veresoglou and Rillig, 2012).

Neotyphodium endophytes exclusively colonize the above-ground parts of their grass hosts, but the consequence is seen in the roots as nematode control. The controlling mechanisms of these endophytes are not fully comprehended as yet. It is suggested that *Neotyphodium* endophytes may alter plant physiology, increase tolerance or resistance to PPNs, or secrete toxic or repellent metabolites which are translocated basipetally into the root (Hallmann and Sikora, 2011). *In vitro* experiments suggested secondary metabolite production as a mechanism for nematode control which is used by endophytic *F. oxysporum*; however, the production of those toxic metabolites in root cells has not yet been proved (Sikora *et al*., 2007). Dababat and Sikora (2007) demonstrated that root exudates from *F. oxysporum*-colonized plants were less attractive to the nematode or they included compounds repellent to *Meloidogyne incognita*. Another possible mechanism of non-pathogenic *F. oxysporum* in suppression of PPNs is competition for nutritious substances and colonization sites, though the involvement of induced systemic resistance (ISR) cannot be ruled out (Hallmann and Sikora, 2011).

2.2.2 Significance in biocontrol of phytonematodes

The biological control potential of various types of nematode-antagonistic fungi has been documented in much research for a long time.

Table 2.1 shows some recent reports on this aspect. The number of fungal-based BCAs of all commercially available biocontrol products (Davies and Spiegel, 2011a; Wilson and Jackson, 2013) indicates the huge significance of fungi for biological control of phytonematodes. For a successful utilization of nematode-antagonistic fungi as a BCA, it is very important to know about their biology at the physiological as well as at the ecological level.

Each group of nematode-antagonistic fungi has its advantages and disadvantages, which affects their significance in biological control. It appears that nematode-trapping fungi may not successfully be introduced as a BCA unless more competent organisms are identified and better delivery systems developed (Chen and Dickson, 2004). Several disadvantages, such as complexity in the establishment in soil, their restricted capturing activity and above all being non-specific to PPNs, reduce their potential in biological control (Moosavi and Zare, 2012). However, new genomic studies can find a way to compensate for those disadvantages (Tunlid and Ahrén, 2011; Yang *et al*., 2011a; Pathak *et al*., 2012; Meerupati *et al*., 2013). The genome of *Arthrobotrys oligospora* strain ATCC 24927 has completely been sequenced. Now this fungus serves as an excellent model organism and will be of more help in comprehending the virulence determinants of this organism with the help of the genome annotation (Niu and Zhang, 2011).

The majority of endoparasitic fungi are obligate parasites and poor saprotrophic competitors in soil, but generally have a wide nematode host range (Moosavi and Zare, 2012). Though this group of fungi possesses no extensive hyphal development outside the body of their prey (Chen and Dickson, 2004), they are more amenable to practical application than nematode-trapping fungi (Persmark *et al*., 1996). Fungi with ingestible conidia cannot infect PPNs since the lumen of the stylet is too narrow to swallow the conidia (Chen and Dickson, 2004), but the fungi with adhering spores are more significant in suppressing PPNs. Some extensively studied endoparasitic fungi are *Drechmeria coniospora*, *Nematoctonus* spp. and *Haptocillium balanoides* (Viaene *et al*., 2006). Though *Haptocillium* is among the most favourable BCA, no commercial product is so far available based on this fungus (Moosavi and Zare, 2012).

At present, and except for zoospore-producing members, egg- and female-parasitic fungi are the most promising group for biological control of economically important PPNs. The zoospores can only be motile in flooded soils; consequently, nematode infection

Table 2.1. List of some fungi with biocontrol potential against phytonematodes.

Fungal species	Nematode	Host	References
Blattisocius dolichus	*Radopholus similis*	Anthurium	Chen *et al*. (2013)
Fusarium oxysporum	*Pratylenchus goodeyi*, *Meloidogyne exigua*	Banana, coffee	Freire *et al*. (2012), Waweru *et al*. (2013)
Pochonia chlamydosporia	*Globodera rostochiensis*, *M. enterolobii*	Potato, guava	Muthulakshmi *et al*. (2012)
Purpureocillium lilacinus	*M. incognita*, *Meloidogyne* sp., *Radopholus* sp., *Heterodera* sp.	Tomato, banana	Mendoza *et al*. (2004), Kiewnick and Sikora (2006a), Ramarethinam *et al*. (2008), Oclarit and Cumagun (2009), Kiewnick *et al*. (2011)
Hirsutella rhossiliensis	*Heterodera glycinea*	Soybean	Wang *et al*. (2009)
Muscodor albus	*M. chitwoodi*, *M. hapla*, *Paratrichodorus allius*, *Pratylenchus penetrans*	Vegetable crops	Riga *et al*. (2008)
Neotyphodium strictum	*M. incognita*	Tomato	Goswami *et al*. (2008)
Trichoderma harzianum, *T. hamatum*, *T. asperellum*	*M. javanica*, *M. incognita*	Tomato	Sharon *et al*. (2001, 2010), Goswami *et al*. (2008)

is limited to periods following rainfall or irrigation. Furthermore, it is difficult, if not impossible, to culture the obligate parasites *in vitro*, therefore their commercial prospect is ambiguous (Graff and Madelin, 1989). Among all nematode-parasitizing fungi, the most frequently isolated species are *Pochonia chlamydosporia* and *Purpureocillium lilacinus* (Morgan-Jones *et al*., 1981, 1983; Rodriguez-Kabana *et al*., 1984; Freire and Bridge, 1985; de Leij and Kerry, 1991; Siddiqui and Mahmood, 1996; Whipps and Davies, 2000; Kerry and Hominik, 2002; Chen and Dickson, 2004; Viaene *et al*., 2006; Moosavi *et al*., 2010; Davies and Spiegel, 2011a, b; Moosavi and Zare, 2012; Wilson and Jackson, 2013). The facultative parasitic members of this group can saprotrophically survive well in the rhizosphere, can be mass-cultured relatively easily and have been found more successful in colonizing their sessile host (eggs, developing juveniles and females). Elucidation of the nematode–fungus interactions at the molecular level, genome sequencing and other '-omics' methods will result in more efficient and effective usage of these BCAs (Rosso *et al*., 2011; Manzanilla-López *et al*., 2012). Production and formulation of filamentous fungal control agents as a commercial product remain challenging (Prabhu *et al*., 2008); however, recent progress in technology has made it possible to produce extremely concentrated formulations that can easily and successfully be used at the field level (Kiewnick and Sikora, 2004, 2006b; Kiewnick, 2006, 2007; Brand *et al*., 2010; Wilson and Jackson, 2013).

New developments in detection, identification and analysis techniques of nematicidal compounds at the cellular and molecular level have opened a novel field of study in biological control. A lot of research has lately been conducted on discovery the pathways of producing compounds with nematicidal activity in toxin-producing fungi. One member of this group, *M. verurrucaria*, is among the few commercially available fungal-based products (Wilson and Jackson, 2013). It is also possible to use the genetically modified organism in biocontrol after the pathways of producing nematicidal compounds are fully understood at the molecular level (Casas-Flores and Herrera-Estrella, 2007).

The disadvantage of AM fungi is that they are only capable of reducing the damage of sedentary and to a lesser extent migratory endoparasitic nematodes; however, these two groups (sedentary and migratory endoparasites) are the two most economically important nematodes among all PPNs. It is also reported that ectoparasitic nematodes impose more damage in the presence of AM fungi (Hol and Cook, 2005), although a contrary report demonstrates that the local and systemic protection against the ectoparasitic nematode *Xiphinema index* has been stimulated in grapevine by an AM fungus (Hao *et al*., 2012). It must be taken into consideration that as a BCA, AM fungi require to be given sufficient time to set up the symbiosis before the plant is infected by the PPNs. Thus, AM fungi are most promising for commercial implementation when they are used in transplanting systems such as vegetables or fruit tree rootstocks. A more clear comprehension of the molecular mechanisms of symbiotic mutualistic association between plants and AM fungi which are responsible for PPN management will hopefully result in novel strategies for better nematode control in the near future (Hallman and Sikora, 2011).

Neotyphodium endophytes are potential BCAs for nematode control, especially in sports greens, as there are several reports of severe damage by PPNs on golf and football grasses. They can be efficiently and inexpensively applied by seed inoculation and, once established, they can vertically be transmitted to next generations via their host seeds (Hallman and Sikora, 2011). Non-phytopathogenic species of *Fusarium* are ubiquitous endophytes and could establish mutualistic association with a large number of plant species. They are extremely pathogenic to nematode eggs as well as secreting metabolites toxic to nematodes. However, more understanding of their controlling mechanisms is required to determine whether *Neotyphodium* or *Fusarium* endophytes can be developed to a commercial BCA or not.

The majority of performed experiments on biological control of PPNs by endophytic fungi has been carried out with non-strictly nematophagous fungi rather than strict ones. Seeing that many nematophagous fungi possess an endophytic phase in their life cycle (Quesada-Moraga *et al*., 2014), the importance of this group in biological control of PPNs

may be altered. For example, it is probable that this ability may provide strictly nematophagous fungi to parasitize eggs inside the roots, to produce trapping structures in the roots, and to serve the fungus as a long-term survival strategy. This may also be a route for evading competition with other soil biota (Macia-Vicente *et al*., 2011). More comprehensive study on optimizing inoculation processes and host-plant growth conditions will lead to the development of appropriate methods for successful exploitation of endophytic growth for PPN control.

2.3 Nematophagous Bacteria

Bacteria numerically represent the most abundant organism in soil, as 1 g of fertile soil contains approximately 10^5–10^8 bacterial colony forming units (Metting, 1993). The bacterial biomass is also very large, as the total weight of bacteria in temperate grassland is estimated to be 1–2 t/ha (Nannipieri *et al*., 2003). Many of these bacteria inhabit the rhizosphere and their environmental modification may result in direct or indirect effects on PPNs as well as on the host–nematode inter-relationship (Neipp and Becker, 1999). Over 99% of the bacteria present in various environmental samples are not culturable (Sharma *et al*., 2005) and consequently still remain unidentified for their ecological functions (Nannipieri *et al*., 2003; Riesenfeld *et al*., 2004). But other culturable ones have been studied extensively for their possible interference with nematode behaviour, feeding and reproduction (Hallmann *et al*., 2009). The nematophagous bacteria are ubiquitous with wide host ranges. They have been isolated from soil, plant tissues, and cysts and eggs of nematodes. A number of these bacteria have already shown great ability in controlling PPNs (Jatala, 1986; Weller, 1988; Sayre and Walter, 1991; Stirling, 1991; Siddiqui and Mahmood, 1999; Kerry, 2000; Walia *et al*., 2000; Dong and Zhang, 2005; Tian *et al*., 2007; Maheshwari *et al*., 2013; Trivedi and Malhotra, 2013).

Some of those bacterial BCAs may cause diseases in humans and must be tested for any adverse effects before registration. The available pathogenicity assays are time-consuming, costly and unsuitable for facultative pathogens. Therefore a novel, quick and inexpensive bioassay was developed on the basis of *Caenorhabditis elegans* (Zachow *et al*., 2009). Nematode-antagonistic bacteria can be grouped into obligate parasites, opportunistic parasites, rhizobacteria, endophytic bacteria and symbiotic bacteria according to their mode of parasitism (Siddiqui and Mahmood, 1999; Tian *et al*., 2007). A number of biologically active bacteria against PPNs are listed in Table 2.2.

The *Pasteuria* group of bacteria are the most-studied bacterial control agent of PPNs. They are obligatory parasites that produce very resistant endospores. The taxonomy of this group of bacteria still remains unclear (Davies *et al*., 2011); however, the results obtained from the most definitive genetic study have placed *P. penetrans* in the low G+C content *Bacillus* group. It appears that this genus belongs to the *Bacillus–Clostridium* clade (Charles *et al*., 2005). Four nematode-antagonistic species of *Pasteuria* have so far been identified: *P. penetrans*, which parasitizes root-knot nematodes (Sayre and Starr, 1985), *P. thornei*, a parasite of lesion nematodes (Starr and Sayre, 1988), *P. nishizawae*, which infects cyst nematodes (Sayre *et al*., 1988, 1991), and *P. usgae*, which infects *Belonolaimus* spp. (Giblin-Davis *et al*., 2003; Preston *et al*., 2003).

Opportunistic parasites commonly refer to saprophytic bacteria that can utilize nematodes as one of their multiple nutrient resources. Similar to obligate parasitic bacteria, opportunistic ones are capable of breaking through the body wall of their prey. *Brevibacillus laterosporus* is considered to possess an opportunistic lifestyle (Tian *et al*., 2007). This bacterium is a ubiquitous species, which is usually present in a large spectrum of environments such as soil, water, invertebrate bodies, etc. It has biocontrol activity against insects, nematodes and molluscs (Ruiu, 2013). Some isolates of *B. laterosporus* could infect plant-parasitic, animal-parasitic and free-living nematodes (Oliveira *et al*., 2004; Huang *et al*., 2005).

Rhizobacteria refer to those bacteria that are capable of colonizing the rhizosphere aggressively (Schroth and Hancock, 1982). Since bacteria are capable of using root exudates as nutrients, their population densities in the rhizosphere are up to 100 times more than in bulk soil (Hallmann *et al*., 2009). The most prevalent nematode-antagonistic rhizobacteria

Table 2.2. List of some bacteria with biocontrol potential against phytonematodes.

Bacteria	Plant nematode	Host	References
Rhizobacteria	*M. ethiopica*, *Xiphinema index*	Grapevines	Aballay *et al*. (2011, 2012, 2013)
Bacillus subtilis	*Aphelenchoides besseyi*, *Ditylenchus destructor*, *Bursaphelenchus xylophilus*, *M. javanica*	Various hosts	Xia *et al*. (2011)
Nitrogen-fixing bacteria, phosphate-solubilizing bacteria, potassium-solubilizing bacteria	*M. incognita*	Various hosts	El-Hadad *et al*. (2010)
Paenibacillus polymyxa, *P. lentimorbus*	*M. incognita*	Various hosts	Son *et al*. (2009)
Pasteuria penetrans	*Meloidogyne* spp.	Vegetables	Davies (2009), Mateille *et al*. (2009)
Bacillus thuringiensis	*M. incognita*	Various hosts	Jouzani *et al*. (2008)
Pseudomonas fluorescens	*M. incognita*, *M. javanica*	Tomato, cotton, cucumber	Hallmann *et al*. (1998), Siddiqui and Shaukat (2003, 2005), Siddiqui *et al*. (2004)
Actinomycetes	*M. incognita*	Various hosts	Chubachi *et al*. (2002)
Enterobacter asburiae	*M. incognita*	Cotton	Hallmann *et al*. (1998)
Stenotrophomonas maltophilia, *Chromobacterium* sp.	*G. rostochiensis*	Potato	Cronin *et al*. (1997)
Agrobacterium radiobacter, *Pseudomonas* spp.	*M. incognita*	Cotton, cucumber	Hallman *et al*. (1998)

are the members of *Bacillus* spp. and *Pseudomonas* spp. (Tian *et al*., 2007). *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Bacillus subtilis* are the most comprehensively studied species (Trivedi and Malhotra, 2013). Other rhizobacterial genera expressing antagonistic potential against PPNs include species belonging to *Actinomycetes*, *Agrobacterium*, *Arthrobacter*, *Alcaligenes*, *Aureobacterium*, *Azotobacter*, *Beijerinckia*, *Burkholderia*, *Chromobacterium*, *Chryseobacterium*, *Clavibacter*, *Clostridium*, *Comamonas*, *Corynebacterium*, *Curtobacterium*, *Desulfovibrio*, *Enterobacter*, *Flavobacterium*, *Gluconobacter*, *Hydrogenophaga*, *Klebsiella*, *Methylobacterium*, *Paenibacillus*, *Phyllobacterium*, *Phingobacterium*, *Rhizobium*, *Serratia*, *Stenotrophomonas*, *Variovorax* and *Xanthomonas* (Siddiqui and Mahmood, 1999; Hallmann *et al*., 2009; Maheshwari *et al*., 2013; Trivedi and Malhotra, 2013). About 1–2% of these rhizobacteria (Antoun and Kloepper, 2001), such as *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Clostridium*, *Desulfovibrio*, *Pseudomonas*, *Serratia* and *Streptomyces*, can improve plant growth and decrease diseases caused by different pathogens, including PPNs. These beneficial microorganisms are called 'plant-growth-promoting rhizobacteria (PGPR)' (Ramamoorthy *et al*., 2001; Antoun and Prévost, 2005). PGPRs may inhabit the rhizosphere, the rhizoplane and the radicular tissues (Beneduzi *et al*., 2013). They can protect their plant hosts against plant pathogens, including PPNs (Lee *et al*., 2013). Actinomycetes, which are capable of producing branching filaments, are another important nematode-antagonistic group (Ruanpanun *et al*., 2010). The members of this group secrete many secondary metabolites, which are of much interest for both industry and medical uses (Schaal *et al*., 2006; Schrempf, 2006). *Streptomyces* is a common actinomycete genus which possesses antagonistic activity against PPNs (El-Nagdi and Youssef 2004; Ruanpanun *et al*., 2010). Species of *Streptomyces* produce a wide range of secondary metabolites with antimicrobial (Hallmann *et al*., 2009) and nematicidal properties (Sun *et al*., 2006; Ruanpanun *et al*., 2010).

Endophytic bacteria inhabit the inner plant tissues with no disease symptoms or strong defence induction. Contrary to endosymbionts, these bacteria do not live in plant cells and they are not enclosed by a membrane compartment either (Stępniewska and Kuźniar, 2013; Reinhold-Hurek and Hurek, 2014). These bacteria have been detected in numerous plant species and in almost all plant parts including above- and below-ground. Most endophytic bacteria may occupy both the rhizosphere and endorhiza simultaneously (Hallmann *et al*., 2009).

Rhizobia are soil inhabiting bacteria belonging to the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Azorhizobium*, and their complex symbiotic association with particular pulse crops results in nodulate roots (Gage, 2004; Damiani *et al*., 2012). They may grow in soil as free-living bacteria until an appropriate host becomes available. A successful establishment of nitrogen-fixing symbionts in root nodule cells may occur only when the rhizobia sufficiently colonize the rhizosphere prior to making contact with the host roots (Trivedi and Malhotra, 2013). These bacteria are of great environmental and agricultural importance since most of the atmospheric nitrogen fixation on earth is attributed to their symbiosis with legumes. In addition to their valuable abilities in nitrogen fixing, which decreases the need for nitrogen fertilizers, they can protect their host from different soil-borne plant pathogens including PPNs (Siddiqui and Shaukat, 2002). Legumes serve as a host for both rhizobia and nematodes; therefore, PPNs can reciprocally influence rhizobial establishment. The effect of nematode infection on plant root nodulation is not always constant as it may cause a decrease (Hussaini and Seshadri, 1975), increase (Hussey and Barker, 1976) or no change (Taha and Raski, 1969) in the amount of nodulation.

2.3.1 Mode of parasitism

Different groups of nematode-antagonistic bacteria differ among themselves in their behaviour, reproduction and biocontrol approaches (Siddiqui and Mahmood, 1999). Among the groups of BCAs, bacteria exhibit the most diverse mechanisms related to parasitism, toxin, antibiotics and enzyme production, competition and induced resistance (Tian *et al*., 2007). In soil, endospores of *P. penetrans* remain dormant and wait for contact with the cuticle of a second-stage juvenile (J_2). Once the contact occurs the endospores adhere to the cuticle and make an entry into the host through the body wall and initiate infection (Bishop, 2011; Davies *et al*., 2011). Sticking of endospores does not necessarily result in infection as it has been observed that endospores become attached to cuticle but fail to parasitize (Davies *et al*., 1990), heat-killed endospores possess the ability of adhering to nematode cuticle (Dutky and Sayre, 1978), and a proportion of spores do not germinate at all (Bishop, 2011). To guarantee a successful infection of a J_2, at least five endospores should bind to its cuticle (Davies *et al*., 1988). In the case of root-knot nematodes, endospore penetration usually occurs after the nematode has set up its feeding site (Chen *et al*., 1996, 1997). Excluding *P. penetrans*, endospore germination of other phytoparasitic bacterial species has been found to occur without the plant interaction (Giblin-Davis *et al*., 2003). Opportunistic bacterial parasites are capable of penetrating the body wall of their prey and adversely affect them by producing secondary metabolites. An extracellular protease was found in certain strains of *B. laterosporus*, which restrains both egg hatching and larval development of nematodes. This compound was a heat stable, low molecular weight protein (Bone and Singer, 1991; Huang *et al*., 2005; Ruiu, 2013).

Rhizobacteria may suppress PPNs by means of one or more mechanisms including direct antagonism through the production of extracellular toxins, enzymes and other secondary metabolites, interfering with host plant–nematode identification, competing for nutrition and rhizobacteria-mediated induced systemic resistance (ISR) (Tian *et al*., 2007). However, rhizobacteria may also indirectly reduce nematode population densities when they decompose the organic matter. Being exposed to decomposition products, such as volatile fatty acids, hydrogen sulfide and ammonia, can result in deleterious effects on nematodes (Walia *et al*., 2000).

PGPRs may directly and indirectly promote plant growth. The direct effect may be provided by supplying the plant with a bacterial synthesized compound, such as phytohormones, or assisting in absorption of certain nutritious substances from the environment. The indirect

growth promotion may occur by secreting antagonistic compounds and induced resistance to pathogens; both result in decreasing the damage of phytopathogenic organisms (Beneduzi *et al*., 2013). PGPRs may adversely alter the nematode behaviour during the early phase of root penetration. They may secrete some metabolites that reduce both egg hatching and host attraction. They may also degrade some root exudates that regulate nematode behaviour (Walia *et al*., 2000). Extracellular metabolites that are secreted by *Streptomyces* species may cause nematode mortality, reduce hatching, or both. The most well-known species with nematicidal activity is *Streptomyces avermitilis*. This bacterium secretes the most potent natural nematicide, called avermectin (Hallmann *et al*., 2009).

Endophytic bacteria are capable of improving plant growth as well as restraining disease development (Tian *et al*., 2007). Both endophytic and rhizobacteria use almost the same mechanisms for enhancing plant growth or suppressing PPNs, regardless of their dissimilar dwelling places. The mechanisms exploited for antagonizing nematodes may lead to competition for food and space, secretion of inhibitory molecules and stimulation of systemic resistance (ISR) in the plant (Hallmann, 2001; Compant *et al*., 2005).

Symbiotic root-nodule bacteria may reduce nematode damage by promoting plant growth or by stimulating changes in their host plants. They assist in plant growth by providing the required nitrogen via fixing atmospheric nitrogen. Rhizobia also induce physiological, biochemical and histopathological changes in their host plant (Trivedi and Malhotra, 2013). Antibiotic and toxic metabolite production ability has been reported for rhizobia (Haque and Gaffar, 1993), which may adversely affect PPNs together with other plant pathogens (Trivedi and Malhotra, 2013).

2.3.2 Significance in biocontrol of phytonematodes

Most of the economically important PPNs are reported to be parasitized by *Pasteuria* spp. (Bird *et al*., 2003). *P. penetrans* evidently has the potential to be developed into a commercial BCA as its association with *Meloidogyne* spp. has been observed in suppressive soils (Oostendorp *et al*., 1991; Weibelzahl-Fulton *et al*., 1996; Trudgill *et al*., 2000; Cetintas and Dickson, 2004), and its infection reduces the fecundity of the females of root-knot nematodes (Davies *et al*., 2008). Members of *Pasteuria* spp. are one of the best known BCAs of PPNs, however, their limited host range (Hallmann *et al*., 2009) (although narrow host range is not always the case, Mohan *et al*., 2012) and difficulties in their mass-production on artificial media together with their slow growth rate (Wilson and Jackson, 2013) have restricted their commercial development so far. Because of the last two reasons, using this bacterium at a large scale will be problematic in the short term. Thus, it will be a rational expectation that the market size of *Pasteuria*-based products will be limited to high-value horticultural crops, transplants and amenity grasses, where complete coverage with high spore densities is feasible (Hallmann *et al*., 2009). To ensure robust control of diverse phytonematode species and populations in different soils, a product may need to contain meticulously prepared combinations of spores with diverse attachment and parasitizing characteristics (Hallmann *et al*., 2009). To date, only one product of this type is commercialized, with the name of Econem™, and that contains *P. usgae*. This product is targeted on sting nematodes (*Belonolaimus* spp.) in turf grass, particularly in golf greens (Wilson and Jackson, 2013). Syngenta announced plans to introduce a *Pasteuria*-based seed-treatment product to control *Heterodera glycines* on soybean in 2014 (Wilson and Jackson, 2013). Genetic modification will certainly be exploited in the near future to increase the possibility of *Pasteuria* spp. for commercial use worldwide.

Opportunistic parasites may be exploited as a formulated effective commercial BCA only if the mechanisms that regulate the switch from saprotrophy to parasitism are fully understood (Tian *et al*., 2007). They may also be exploited for their extracellular products that adversely influence nematodes, with the advantage of being grown saprophytically.

To date, several rhizobacteria-based products are available in the market in spite of lower control potential of rhizobacteria under field conditions. For successful nematode suppression, it is vital that bacterial controlling

agents occupy the rhizosphere prior to other microorganisms. Early rhizosphere colonization can be ensured by either seed treatment or soil drench instantly following seeding. It must be taken into consideration that concurrent application of two or more rhizobacteria frequently results in lesser control because of competition (Hallmann *et al*., 2009). Commercially available rhizobacteria-based nematicides have been developed for *Burkholderia cepacia*, *Paenibacillus macerans* and *Bacillus amyloliquefaciens* (Trivedi and Malhotra, 2013) and *Bacillus firmus* (Wilson and Jackson, 2013). Another commercial bionematicide on the market has been produced based on *S. avermitilis*, with the trade name Avicta®, and is being used as a seed treatment (Hallmann *et al*., 2009).

Endophytic bacteria have been shown as potent antagonists; however, little is known about the biology and ecology of these organisms when used as a BCA. We also know very little about the precise mode of action and their potential importance for nematode suppression in large-scale experiments. Certainly, endophytes will be subjected to further studies in the near future because of their resemblance to rhizobacteria (Hallmann *et al*., 2009) along with the fact that several rhizobacteria-based commercial products are now available in the market (Wilson and Jackson, 2013). These make endophytic bacteria fascinating organisms for biological control with a bright prospect. However, further complementary experiments on this group may need prior development of specialized methodologies.

As a plant growth enhancer, symbiotic root-nodule bacteria, PGPRs or endophytic bacteria can be used for both better yield and possible disease control. No registered product for nematode control has been developed on the basis of these groups of bacteria. There are several products sold as plant growth promoters, soil conditioners, biofertilizers and biological activators, but they do not meet registration processes for nematode control. Therefore, application of those products as BCAs will continuously be associated with an unestimated level of danger because no experiment has been carried out on toxicology, environmental impact and persistence of effect (Whipps and Davies, 2000; Whipps and Lumsden, 2001; Ehlers, 2011). A comprehensive understanding of nodule formation and functioning at both the cellular and molecular levels is of considerable interest, because a successful commercial implementation against PPNs may occur when these mechanisms are fully elucidated.

2.4 Predatory Nematodes

In spite of a hundred-year-old interest in using predaceous nematodes as a BCA (Cobb, 1917), their capability has only begun to be studied in recent years. Among a broad range of soil predators which prey on nematodes, predatory nematodes are the most important (Bilgrami and Brey, 2005). They eat all types of nematodes and have established themselves as an important part of the soil food web. In addition to their biocontrol potential against PPNs, they stimulate cycling of plant nutrients, which in turn may help plants better withstand any nematode damage (Yeates and Wardle, 1996; Stirling, 2011).

Predaceous nematodes mainly belong to the orders Mononchida, Rhabditida (infraorders Diplogasteromorpha, Rhabditomorpha; and superfamily Aphelenchoidea), Dorylaimida (superfamilies Dorylaimoidea, Nygolaimoidea, Actinolaimoidea) and Enoplida (families Ironidae, Oncholaimidae, Monohysteridae and Thalassogeneridae).

2.4.1 Mode of parasitism

Each group of predaceous nematodes possesses diverse feeding apparatus, and also differs in behaviour (prey searching and/or catching), food preferences and feeding mechanisms (Khan and Kim, 2007; Bilgrami, 2008). The buccal cavity of the mononchid predators is highly sclerotized with strong musculature, a big piercing dorsal tooth and small grasping teeth or denticles. Such feeding apparatus enables them to swallow their prey when it is of smaller size, or cut their larger prey into pieces. The second group, which is usually called stylet-bearing predaceous nematodes, is composed of dorylaimids, nyglolaimids and aphelenchids (Bilgrami and Brey, 2005). The feeding apparatus of these predators is of a piercing and sucking

type, which enables them to perforate the body wall of their prey and suck the body contents. Dorylaimid predators possess a hollow stylet called the odontostyle, while feeding apparatus in nyglolaimids is large, solid and slender, with a protrusible tooth called the mural tooth. Aphelenchid predators have a fine needle-like stylet with a lumen for ingestion. Dorylaimid predators use their long hollow stylet to disrupt the interior organs of the prey to render them motionless very quickly (Linford and Oliviera, 1937), whereas aphelenchid predators use their stylet to insert digestive enzymes into the prey body and paralyse it (Hechler, 1963; Wood, 1974). The nyglolaimids' tooth has no lumen, therefore it is solely used for piercing or slitting their prey and ingesting the prey body contents (Khan and Kim, 2007). The third feeding type that is represented by diplogasterids is the cutting and sucking style. The buccal cavity of the diplogasterid predators is small but well equipped with a strong claw-like movable dorsal tooth (Khan and Kim, 2007). They may possess teeth or denticles for cutting the cuticle of the prey or crushing the food particles (Jairajpuri and Bilgrami, 1990).

2.4.2 Significance in biocontrol of phytonematodes

Some critical characteristics determine the commercial success of predatory nematodes as potential BCAs. Mass productivity at a commercial scale and on a cheap substrate is a primary trait. Compatibility with agrochemicals and routine farm measures, possessing efficient prey searching ability together with rapid dispersal capacity, safe to non-target organisms, endurance to fluctuation of abiotic environmental factors, high reproducibility along with high infective potential, and considerable durability and stability are the important characteristics. Ability to quickly reduce PPN population densities as well as producing toxic metabolites are other desirable traits (Bilgrami, 2008).

Predatory nematodes are not considered as a viable choice for controlling PPNs since they are not commercialized yet. It seems that they have a long way to go to pass all necessary experimental tests in their commercialization process. Among the different groups of predators, diplogasterids seem the most probable candidate to be sold in future as a commercial product, as they satisfy many requirements of being a good PPN predator. Their advantages are short life cycle, easy culture, prey-specificity, chemotaxis sense and resistance to adverse conditions (Bilgrami and Brey, 2005; Khan and Kim, 2007). The dorylaimid predators may possibly be the second best candidate, but their long life cycle is a great deterrent. However, only additional research will confirm their effectiveness at commercial level (Bilgrami, 2008).

2.5 Predaceous Mites

The first report on probability of controlling PPNs by mites was by Linford and Oliveira (1938). Six (undetermined) mites collected by them were found to be potential BCAs for *Meloidogyne marioni* in Hawaiian suppressive soils. Since then various mites have been identified that feed on nematodes. It is now obvious that in soil ecosystems, nematodes are a preferred food regime for many mites (van de Bund, 1972; Muraoka and Ishibashi, 1976; Walter *et al*., 1987; Walter and Ikonen 1989; Stirling, 1991; Bilgrami, 1994; Koehler, 1999; Gerson *et al*., 2003; Beaulieu and Walter, 2007; Pakyari and Maghsoudlo, 2011; Walter and Proctor, 2013). The mites that feed on nematodes belong to several acarine cohorts. Taxonomy of important nematophagous mites is shown briefly in Table 2.3.

2.5.1 Mode of parasitism

The body structure of nematophagous mites is adapted to their lifestyle. They are small, elongate, flexible and/or have the ability of extruding their chelicerae considerable distances into small soil pores or into water film around the soil particles to hunt their prey. These predators can survive periodic flooding and most of them have the ability of seeking for nematodes, or even protozoans, within a water layer (Walter and Proctor, 2013).

Nematophagous mites can be categorized into three functional groups according to their dependence on nematodes as a food supply

Table 2.3. Taxonomy of the most common nematophagous mites.

Superorder	Order	Suborder	Family	Remarkable genus
Acariformes	Sarcoptiformes	Astigmata	Acaridae	*Tyrophagus* spp.
		Oribatida	Ceratozetidae	
			Galumnidae	*Pergalumna*
			Haplozetidae	
		Endeostigmata	Alicorhagiidae	*Alicorhagia*
			Alycidae	*Alychus*
			Eupodidae	
	Trombidiformes	Prostigmata	Paratydeidae	
			Tydeidae	
Parasitiformes	Mesostigmata	Dermanyssina	Ascidae	*Gamasellodes* and *Lasioseius*
			Digamasellidae	*Dendrolaelaps*
			Eviphididae	
			Laelapidae	*Geolaelaps*
			Macrochelidae	*Macrocheles*
			Ologamasidae	
			Phytoseiidae	
			Rhodacaridae	*Rhodacarus*
			Veigaiidae	
		Parasitina	Parasitidae	*Pergamassus*
		Epicriina	Zerconidae	
		Monogynaspida	Uropodidae	

(Walter *et al*., 1988). The first group, called general predators, has no food preference. Most nematophagous mesostigmatids belong to this group since they have the ability to consume any encountered prey as well as decomposing vegetation, compost, dung and carrion. However, the growth parameters (rapid development, higher fecundity and lower mortality) of these mites improve when fed nematodes (Walter *et al*., 1987; Walter and Ikonen, 1989). Mesostigmatids only ingest the body fluid of their prey nematode by piercing and crushing their body wall (= fluid feeders) (Gerson *et al*., 2003).

Oribatid mites are general predators with a certain degree of specialization with regard to their food resources (Schneider *et al*., 2004). The predatory behaviour of many oribatid mites on nematodes has been confirmed using molecular methods (Heidemann *et al*., 2011). They typically grab the prey nematode at one end and pull the entire body into the oral cavity (= engulfers) (Gerson *et al*., 2003). Other general feeders are found in the Laelapidae, Parasitidae, Rhodacaridae and Ologamasidae (Walter and Proctor, 2013).

Fungivorous mites are the second group that can consume algae and nematodes as well. This group comprises the Acaridae, the Ceratozetidae and many prostigmatids (like Tydeidae). Specialized nematode predators that prefer nematodes or that feed only on them compose the third group which include the families Ascidae, Eviphididae, Macrochelidae, Uropodidae, Zerconidae, Eviphididae and Alicorhagiidae. Several genera of laelapid mites (e.g. *Laelaspis* and *Cosmolaelaps*) are also specialized nematophages (Gerson *et al*., 2003; Walter and Proctor, 2013). The mites of the third group are not exclusive feeders on PPNs and there is also no report on successful management of phytonematodes by these specialists (Gerson *et al*., 2003).

2.5.2 Significance in biocontrol of phytonematodes

Relationship between mites and nematodes is a long-neglected aspect of soil ecology, which is mainly caused by the disciplinary disjunction between soil nematology and acarology. Soil nematology is historically placed within the plant pathology discipline rather than zoology. As a result, when plant pathologists are looking for BCAs of nematodes, they involuntarily

notice the microbial control agent or predatory nematodes. Soil zoologists frequently also ignore nematodes in their ecological studies (Walter and Proctor, 2013).

Only the members of the general feeders and fungivorous groups have been considered as potential BCAs of pest nematodes, regardless of their lack of specialization. Although feeding on other diets may decrease their importance as a nematode-consumer, it apparently increases their longevity in the absence or shortage of their prey nematode. Moreover, the non-specialized predators are prevalent in the soil and are often very voracious (Gerson *et al*., 2003; Walter and Proctor, 2013). Despite the presumable role of mites in the regulation of nematode populations in natural ecosystems (Stirling, 2011), their significance in biocontrol of PPNs in arable lands is arguable. Notwithstanding many rapidly growing publications from 1970 to 1995 about the worth of mites as a potential BCA of PPNs, only a few articles have recently been published on this topic. Little information is available about predation rate of mites against PPNs in agricultural soils, because the existing knowledge was mainly obtained from laboratory or greenhouse studies that need to be validated under field conditions. However, difficulties in mass production of mites and their delivery to soil (Viaene *et al*., 2006) have faded the hope of using mites as a commercial BCA. Furthermore, non-specificity to PPNs makes the mites inappropriate for use in biological control programmes aimed at specific nematode pests. These obstacles make the mites impractical as a successful BCA against phytonematodes. However, the increase in our knowledge on soil ecology and innovative progress in mass production and delivery technology may rekindle the interest in mites in the near future.

2.6 Viruses

The pathogenic impact of viruses on nematodes is not very clear; however, development of insect viruses to commercial products (Falcon, 1976; Carter, 1984; Hunter-Fujita *et al*., 1998; Flexner and Belnavis, 2000; Sun and Peng, 2007; Erlandson and Theilmann, 2009; Burand *et al*., 2009; Kamita *et al*., 2010; Rodriguez *et al*., 2012) along with similarity of nematodes to insects, which causes both of them being placed in the same superfamily Ecdysozoa (Telford *et al*., 2008), strengthened the concept of using nematode viruses as a feasible control measure. Several species of PPNs are natural vectors of plant viruses without being infected (Brown *et al*., 1995; Hull, 2009). In addition, there are several reports on viruses that infect nematodes and replicate within their nematode hosts. Loewenberg *et al.* (1959) were the first to report a nematode disease, presumably caused by a virus, which made *M. incognita* (J_2) extremely sluggish. The body of infected juveniles appeared to be extremely vacuolated or filled with unusual oil-like globules and they were incapable of forming galls. The suspension of sluggish nematodes was found to transmit the disease even after it passed through bacterial filters, and maintained its virulence following serial passages. The disease was supposed to be caused by a virus; however, the probable involvement of a mycoplasma was not eliminated because the virus particles were not detected in diseased nematodes. Foor (1972) observed cellular abnormalities in both males and females of *Trichosomoides crassicauda* in which virus was supposed to be involved. The nuclei of somatic cells in infected nematodes included some spherical virus-like particles with a diameter of 15 nm. Similarly, some polyhedral virus-like particles with a diameter of 20 nm were detected in cytoplasm of intestinal cells of *Dolichodorus heterocephalus* (Zuckerman *et al*., 1973).

Another report on abnormal behaviour of nematodes is related to the phenomenon of swarming (aggregation of nematodes in masses) in *Tylenchorhynchus martini*. The swarmers appeared to be more susceptible to chemicals and other unfavourable conditions. Some symmetrical virus-like inclusion bodies were detected in the internal tissues and on the surface of the cuticle of swarming nematodes rather than healthy ones. Likewise, partial disintegration from epicuticle to median zone was only found in swarming nematodes (Ibrahim, 1967; Ibrahim and Hollis, 1973). The exact causal agent of the swarming disease remained unknown as it was not transmittable from swarming to non-swarming nematodes (Ibrahim *et al*., 1978). There is also a report

that claimed replication of an Iridovirus in a mermithid insect-parasitic nematode (*Thaumamermis cosgrovei*) and its insect hosts, *Porcellio scaber* and *Armadillidium vulgare* (Poinar *et al*., 1980). Some virus-like particles were observed in *Eutobrilus heptapapillatus* which appeared to be associated with a disease that resulted in tissue breakdown. The infected nematodes had large crystalloid bodies in their pseudocoeloms along with numerous particles resembling icosahedral viruses, in all parts of the nematode except for the crystalloids and the cuticle (Bird *et al*., 1991). Recently, three nodaviruses that could infect and replicate in *C. elegans* and its close relative, *C. briggsae*, have been discovered and their genome has been completely sequenced (Félix *et al*., 2011; Franz *et al*., 2012; Guo and Lu, 2013). These distinct viruses, one specific for *C. elegans* (Orsay virus) and two for *C. briggsae* (Santeuil virus and Le Blanc virus), were isolated from natural populations of those nematodes, which cause abnormal morphologies of intestinal cells. These viruses are similar to those of the Nodaviridae family, with a small, bipartite, positive-sense RNA genome. Infection of the nematode hosts by each virus only occurred by horizontal transmission rather than transovarial transmission (Félix *et al*., 2011; Franz *et al*., 2012). The family Nodaviridae is currently subdivided into two genera: *Alphanodavirus*, which infects insects, and *Betanodavirus*, which infects fish. The members of this family are small (28–37 nm), non-enveloped, icosahedral and typically have packaged bipartite positive-sense RNA genomes (Venter and Schneemann, 2010; Thiéry *et al*., 2012).

Four different negative-sense single-strand RNA viruses are reported to infect eggs and J_2 stage of soybean cyst nematode (SCN), *H. glycines*. These viruses are designated as SCN nyavirus (ScNV), SCN rhabdovirus (ScRV), SCN phlebovirus (ScPV) and SCN tenuivirus (ScTV). They are distantly related to nyaviruses and bornaviruses, rhabdoviruses, bunyaviruses and tenuiviruses, respectively. The epidemiology and exact mechanism of viral transfer among SCN populations is unknown, but the presence of these viruses in both eggs and J_2 of SCN proposes a trans-ovarial transmission. However vertical movement of the virus could rationally be an efficient means of transfer for a soil-borne endoparasitic nematode. Some other members of the virus families to which the novel viruses are related, can replicate in or be vectored by insects, as well as cause important diseases in plants and animals (Bekal *et al*., 2011).

2.6.1 Mode of parasitism

There is little information available about the ways the viruses infect PPNs. Phytonematodes are microscopic in size and some economically important groups typically reproduce and feed within plant roots (Perry and Moens, 2013). Therefore it is quite difficult to monitor or extract the parasitic stages free of plant material and in quantities large enough for detection of probable virus symptoms (Bekal *et al*., 2011). Viruses can cause abnormalities in body structure or nematode behaviour. However, by using traditional nematode extraction methods, detection of any other virus symptoms is unexpected. Apart from the centrifugal flotation method, other nematode extraction techniques do not recover slow-moving or immobile ones (van Bezooijen, 2006), however collecting large numbers of infected nematodes even by centrifugal flotation method is impractical. Furthermore, it is really too hard to microscopically distinguish virus-infected nematodes from those that are diseased or inactive for other reasons (Mankau, 1981). Therefore, it is not an exaggeration to say that detection of any viral pathogens in nematodes depends on fortuitous situations unless genomic approaches for viral discovery are utilized. New methods of high-throughput DNA sequencing enable researchers to quickly identify unknown viruses from earlier recalcitrant organisms and environments (Edwards and Rohwer, 2005; Culley *et al*., 2006). By these methods, comparatively low amounts of starting material could yield huge amounts of data. Moreover, many possibly infected individuals could be inspected in a single experiment (Ansorge, 2009).

It was only recently that viruses were documented as being detected in nematodes (Bekal *et al*., 2011; Félix *et al*., 2011; Franz *et al*., 2012). These discoveries may be of much help in understanding the ways that these viruses

influence the fitness of the nematode, strategies of viral transmission, viral epidemiology in populations, mechanisms of viral pathogenesis and dynamics of nematode–virus interactions. Finding viruses in *C. elegans* and *C. briggsae* has facilitated the study of virus–host interactions by investigating the virus behaviour in these two well-established model organisms. Viral proteins and RNAs were more recently detected in one to six of the 20 intestinal cells present in *Caenorhabditis* nematodes. These findings may be helpful to enhance our perception about viral protein expression in other nematode hosts (Franz *et al*., 2014).

2.6.2 Significance in biocontrol of phytonematodes

It is rather difficult to judge the significance of viruses for biological control of phytonematodes, since studies on this aspect are too few to draw any reasonable conclusion. Inadequate information on nematode-infectious viruses is surprising as nematodes are the second most numerous animals on earth (Perry and Moens, 2013). This knowledge shortage might be as a result of difficulty in extraction or culturing large numbers of infected nematodes needed for virus characterization (Bekal *et al*., 2011). The incidence of pathogenic viruses among PPN populations seems possible because viral pathogens can infect a large number of organisms. Until recently, there were no unequivocal reports on proving viral pathogens in nematode populations. However, natural viruses that could infect and replicate in nematodes have now been documented (Bekal *et al*., 2011; Félix *et al*., 2011; Franz *et al*., 2012). These findings support the assertion that viruses may be common but overlooked in nematodes (Félix *et al*., 2011).

Infection of the nematode by viruses occurs by both horizontal (Félix *et al*., 2011) and vertical (Bekal *et al*., 2011) transmission. Horizontal transmission occurs among migratory nematodes while vertical transmission occurs among sedentary nematodes. Thus, close proximity of nematode individuals to each other is necessary for horizontal transmission. PPNs may contact each other at the root surface or even in the soil environment, when an adult male nematode mates with the female (Bekal *et al*., 2011). Aggregation behaviour of many nematodes that is considered a survival mechanism (Wang *et al*., 2009) may be another way of transmission.

Discovery of natural viruses in *C. elegans* has changed this nematode to an attractive model organism for studying virus–host interactions and a great deal of effort is now being spent in analysing the host responses to infection at histological, cellular and molecular level. The results of these studies may elucidate some more aspects of viral disease such as their transmission, survival, epidemiology, infectious patterns and the cycle of infection, which would ultimately help in implementation of viruses or derivatives thereof for managing PPNs.

2.7 Conclusions

There has been an increase in the number of research studies dealing with biological control of PPNs in the past few decades and this will continue to flourish because of the concurrent need to preserve the environment and promote human health in the society. In soil, PPNs are subjected to parasitism by many natural antagonists including fungi, bacteria, predatory nematodes, mites, viruses, protozoa, insects, etc. (Stirling, 2011), however, the significance of each group is not the same. Nematode-suppressive soils are the best place for finding potent natural enemies (Timper, 2011). Selection of a qualified BCA is the first step in the commercial development process, which has been performed many times in various geographical locations. But most of those BCAs do not meet commercial success because of problems in their mass production, formulation, stabilization and delivery technology. Fungi and bacteria are the most eligible BCAs of phytonematodes, however other BCAs are also receiving attention from researchers at present.

Fungi have received the most attention among the many microorganisms that feed on nematodes partly because of our extended knowledge on their biology and ecology and partly because of improved techniques in formulating and delivering fungal BCAs into the soil. Genome sequencing and genetic engineering

are available techniques to improve the pathogenicity and survival of nematode-antagonistic fungi. More understanding of the biology, ecology and metabolism of BCAs at molecular level will certainly lead to their more successful exploitation (Manzanilla-López *et al*., 2012). At present, egg- and female-parasitic fungi are the most promising group for biological control of economically important PPNs. However, recent studies have revealed the importance of other groups (such as toxin-producing and endophytic fungi) too, which had been overlooked previously. Future progress will definitely solve the existing obstacles in the commercialization path of these potential BCAs.

Next in importance to fungi are bacteria, as they have diverse mechanisms such as parasitism, toxin, antibiotics and enzyme production, competition and inducing resistance. Several rhizobacteria-based products are now available in the market. Endophytic bacteria have also proved to be influential antagonists, however their commercial use may be lagged until more information is received regarding their biology, ecology, mode of action and importance in nematode suppression on a large experimental scale. One important advantage of bacterial BCAs is their simple genome, which eases their biotechnological interventions.

The importance of predatory nematodes in nematode management has not been paid much attention, as no commercial product has been developed from this group of BCAs so far. Biocontrol significance of predatory nematodes differs with their type. The substantial probability of applying predaceous nematodes against phytonematodes lies in the diplogasterids, since they possess some prerequisites of an efficient BCA.

The significance of mites in biocontrol of PPNs in arable land is ambiguous. The problems involved in their mass production and introduction to the soil as well as their non-specificity to hosts make the use of mites impractical in the management of phytonematodes. However, increase in our knowledge on soil ecology, and innovative progress in both mass production and delivery technology may refresh the interest in mites as a potential BCA of phytonematodes.

It is now really hard to define the significance of viruses as a BCA from the facts presented on viral diseases of nematodes. There is very little information on nematode-infectious viruses but additional studies may yield data that would be valuable for biological control. Currently, high-throughput DNA sequencing enables researchers to identify quickly unknown viruses from different organisms. Natural viruses that could infect and replicate in nematodes are now documented in both free-living and PPNs. These findings support the assertion that viruses may be common but ignored in nematodes and can be used in nematode management.

It is a fact that biological control will not replace chemical nematicides, instead it will remain one of the significant components of nematode disease management. Further research is needed particularly on the mechanisms of action of nematophagous fungi and bacteria in order to harness their efficiency and effectiveness in managing PPNs in agriculture. Consequently, this will hopefully provide more commercialized biocontrol products available for PPNs in the market.

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Part II

Nematophagous Fungi

3 Nematophagous Fungi as Biocontrol Agents of Phytonematodes

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3.1 Introduction

Plant parasitic nematodes are recognized a serious threat to crop production throughout the world. They cause significant damage to field crops (Luc *et al.*, 2005), fruit and horticultural trees (Askary *et al.*, 2000; Askary and Haider, 2010). All crop plants are susceptible to at least one nematode species and it is considered that the damage potential of nematodes exists in all climates on any crop (Bridge and Starr, 2007). Globally, agricultural losses due to plant parasitic nematodes have been estimated at US$358 billion annually (see Abd-Elgawad and Askary, Chapter 1, this volume). Plants infected with nematodes are often overlooked and mis-diagnosed as the symptoms shown by the plants are not clear and are very much similar to fungal diseases or nutritional disorders. In some cases crop yield suppression occurs prior to the expression of explicit disease symptoms. The extent of damage caused to plants by these tiny creatures varies with the genera and species (Askary *et al*., 2012).

In recent years management of plant parasitic nematodes has been accomplished primarily through chemical nematicides, crop rotation and resistant cultivars wherever available (Widmer and Abawi, 2000). However, public awareness about the environmental hazards, high cost of chemical nematicides, limited options of crop rotation and availability of very few resistant cultivars requires the development of new management strategies. Under these circumstances, an alternative method in the management of plant parasitic nematodes seems to be biological control where the use of beneficial or antagonistic microorganisms can suppress soil-borne pathogens in soil (Berg *et al.*, 2005). Biological control is considered the most relevant and least damaging approach as it is ecofriendly, economically viable and offers a sustainable and cost-effective alternative to chemical nematicides (Shamalie *et al.*, 2011). It can be either: (i) natural; or (ii) induced. In the former case a natural population of a particular organism inhibits the growth and development of nematodes whereas in the latter case biocontrol agents (BCAs) are introduced artificially (Brand *et al.*, 2010). Biological control may be defined as the reduction of nematode populations through the action of living organisms other than the nematode-resistant host plant, and which occurs naturally, or through the manipulation of the environment or the introduction of antagonists (Stirling, 1991). The aim is to maintain the nematode population below the economic threshold level, rather

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than eliminating it as is done by the chemicals. Hence, biological control is technically a reduction in nematode population accomplished through the introduction of antagonists or manipulation of the environment in order to make it congenial for the activity of naturally occurring antagonists.

Numerous natural enemies attack nematodes and reduce their population, however, the most promising and practicable BCA which regulates the plant parasitic nematode densities in soil is nematophagous fungi. There is a diverse group of antagonists of nematodes that infect, kill and digest their hosts (Nordbring-Hertz *et al*., 2006). Nematophagous fungi are unique in that they interrupt the nematode by utilizing certain structures meant for attacking nematodes. Due to their predaceous and parasitic activities, these carnivorous fungi occupy an important position among the microorganisms that regulate nematode densities in soil (Ehteshamul-Haque *et al*., 1994; Whipps, 1997, 2001; Regaieg *et al*., 2010). They use special mycelial structures, i.e. traps for trapping vermiform moving nematodes or spores for attaching on the nematode cuticle or hyphal tips for attacking nematode eggs, females and cysts (Stirling, 1991). *Pochonia chlamydosporia* (Escudero and Lopez-Llorca, 2012), *Paecilomyces lilacinus*, *Trichoderma harzianum*, *Arthrobotrys oligospora* (Sharma and Pandey, 2009) and *Aspergillus niger* (Li *et al*., 2011) are important fungi which parasitize nematodes and decrease their population. They are widely distributed in almost all types of soil, and are abundant on plant root surface, leaf litter as well as decaying organic matter (Khan, 1990, 1998). The degree of predacity varies with the species and types of nematodes. There are more than 200 species of nematophagous fungi, which differ in saprophytic or parasitic ability (Nordbring-Hertz *et al*., 2011). Research on nematophagous fungi began with the first observation on endoparasite *Harposporium anguillulae* (Lohde, 1874). Since then many of the fungi have been found to feed on nematodes; the list of the fungi known to have adopted this mode of life has been steadily growing. Invention of some chemical pesticides and their commercialization from the 1940s onwards sidelined biological control. The approach of biological control, however, was revived when failure of pesticides and their ill effects were realized. In the past three decades there has been growing attention among research practitioners about the exploitation of these beneficial fungi in the management of plant parasitic nematodes. The commercial products of these fungi in the form of biopesticides have been developed, which have shown promising results in the management of phytonematodes, providing early protection to crop plants (Moosavi and Zare, 2012; Singh, R.K. *et al*., 2013; Singh, U.B. *et al*., 2013).

The present chapter aims to highlight the significance of nematophagous fungi, their efficacy as BCA as well as their possible role in future control strategies of nematodes, particularly those concerning the integrated nematode management (INM) programme.

3.2 Nematophagous Fungi

In the biotic community of soil, there are various levels of ecological relationships. A number of plants fulfil the role of predator on the animal kingdom. These are recognized as plant carnivores that trap, kill and consume living animals. They are microscopic, deadly to their animal prey and can be found in great profusion and variety in any pinch of soil and garden compost. Among these natural groups, one is carnivorous fungi, which utilizes various devices to kill and consume the nematodes and are commonly known as nematophagous fungi or nematode-destroying fungi. Soil population of these nematophagous fungi and their biodiversity is dependent on the presence of organic matter as well as nematodes in the soil (Singh *et al*., 2006). Under microbial management of phytonematodes, research is being concentrated on the isolation and selection of indigenous nematophagous fungi. Use of local isolates of these interesting fungi have shown promising results against phytonematodes and is receiving considerable attention in many developed and developing countries. Nematophagous fungi are an interesting ecological group of fungi responsible for keeping the nematode population in check and are an important part of the subsoil ecosystem. The nematodes are preyed on by

the fungi and the fungi in turn are consumed by organisms on the next trophic level. An interesting feature of this predator–prey relationship is that the nematodes like many other soil inhabitants secrete chemicals while passing through the soil, which are detected and responded to by these carnivorous fungi and accordingly they act to ensnare the nematodes by developing certain trapping devices. Mankau (1980) stated that the nematode-destroying fungi play a major role in recycling the carbon, nitrogen and other important elements from the rather substantial volume of nematodes that browse on microbial primary decomposers. Species of nematophagous fungi belong to many taxonomic groups, which include oomycetes, chytridiomycetes, zygomycetes, ascomycetes, basidiomycetes and deuteromycetes. According to the type of pathogenesis in nematodes, nematophagous fungi are usually divided into endoparasites and predators (Barron, 2004), however, on the basis of their modes of attack, nematophagous fungi can be classified into four groups: (i) nematode-trapping fungi using adhesive or mechanical hyphal traps; (ii) endoparasitic fungi using their spores; (iii) egg- and female-parasitic fungi invading nematode eggs or females with their hyphal tips; and (iv) toxin-producing fungi immobilizing nematodes before invasion (Barron and Thorn, 1987; Dackman, *et al.*, 1992; Liu *et al.*, 2009). This chapter will start by describing the method of isolating these nematophagous fungi from soil in general, their observation under a microscope, preparation of slides and identification. Finally, each group of fungi will be discussed separately.

3.2.1 Isolation techniques

In the soil ecosystem, nematophagous fungi interrupt the nematode by utilizing certain devices for capturing and killing nematodes. Procedures for obtaining and observing this unusual phenomenon of nature have been described by few research workers (Duddington, 1955b; Barron, 1969) and require some specialized technique, although some patience and careful observation is required to explore them in soil samples.

Srivastava (1987) described the soil sprinkling technique to isolate nematophagous fungi, which in the author's opinion is an easy method to isolate the nematophagous fungi from soil. While collecting the soil sample care should be taken that the sample should be from a cultivated area or irrigated field and not from barren land. It is better to collect the soil sample from the upper 10–20 cm of soil because the presence of plant parasitic nematodes is more likely there, which increases the chances of the presence of these carnivorous fungi. On the same or subsequent day of collection the samples are processed for the isolation of nematophagous fungi. Using standard aseptic technique the samples are unpacked and sprinkled on a sterilized petri plate having 2% agar media (water agar media). Approximately 1–2 g soil is sprinkled over the agar plate and then inoculated with 1 ml of nematode suspension obtained from the same soil sample by Cobb's sieving and decanting method (Cobb, 1918) after concentrating the nematode suspension in 5 ml water. Nematode suspension is added to the plate within 48 h after the soil is sprinkled. The petri plate thus prepared is left to incubate at room temperature between 22 and 28°C. The sprinkling of soil is done with the use of a sterile scalpel or forceps, which is sterilized after each operation.

3.2.2 Observation of living materials

Primary or mixed culture is ready for observation after the initial growth of fungi covers the surface of the agar. At the time of observation under a microscope, the plate can be uncovered without serious contamination. It has been observed that the time taken for the fungi to appear may be anything from 1 week to 2 months. Zoopagales preying on nematodes appear first followed by the monoliales group. Primary examination is best carried out with a low magnification stereoscopic microscope. The soil plates are scanned on alternate days at 100X magnification for trapped or infected nematodes. Inspection of actual haustoria of the fungi is essential to confirm the mycelium belong to trapping fungi. Some of the nematophagous fungi are short lived, therefore the

culture must be inspected at least at an interval of 2 days and more often if possible. A small piece of agar bearing the specimen is cut out and the greater part of the lower portion of agar is sliced away with a sharp razor blade under a stereoscopic microscope and placed on a new agar plate bearing the nematode for further examination or placed on a slide with the help of lactophenol and stained with very light cotton blue for the preparation of a temporary slide. Regular examination is necessary and if one of these fungi is found, its position is marked by pressing the point of a marker pen against the glass bottom of the plate.

3.2.3 Temporary and permanent mounts

Duddington (1955b) described a technique for making permanent mounts. A good permanent preparation for nematophagous fungi is not easy to make and is less satisfactory to study than water mounts of the living material. The endoparasitic fungi are an exception as they mount well in lactophenol and cotton blue. This unfortunately gives a poor result with the nematode-trapping hyphomycetes and is worthless for a member of the zoopagales. However, they can be mounted in cotton blue and lactophenol for a few months.

3.2.4 Identification

Literature on nematophagous fungi belonging to various groups is fragmentary and not compact to date, however, a majority of known species have been described by Drechsler and Barron. Identification is made directly at 400X magnification. The fungus is further cultured from the mixed soil plates either by picking up the conidia with a sterile agar-dipped needle from raised conidiophores above the surface of the agar, or by cutting the agar block under a binocular microscope and planting on baited water agar plates for re-culturing the fungus whenever required for further examination. The key provided by Cooke and Godfrey (1964) is based on trapping mechanism, morphology of conidia and spores and therefore it proves very helpful in the identification of nematophagous fungi, although the original description should always be consulted for confirmation of all identifications.

3.3 Nematode-trapping Fungi

Nematode-trapping fungi are soil-borne microfungi that possess sessile trapping devices to entrap moving stages of nematodes. Such fungi form traps in the presence of nematodes. The trapping structures produced are of various shapes and sizes and the entrapment takes place when the nematodes migrate to the trap and the trap attaches to the nematode surface. The nematodes thus trapped are killed and digested by the fungus. These fungi are not host specific and therefore all soil-dwelling nematodes are the victims of such fungi. Different trapping devices are produced by these carnivorous fungi such as adhesive knobs, adhesive hyphae, constricting rings, and two-dimensional or three-dimensional adhesive networks (Table 3.1).

3.3.1 Taxonomy and morphology

Research on nematode-trapping fungi began in the first half of the 19th century and the first genus described was *Arthrobotrys* (Corda, 1839). Later on in 1852, Fresenius described the first species of this genus as *A. oligospora.* Drechsler published his first paper on predatory fungi in 1935 and continued until 1962 with their isolation and investigation, describing more than 100 new species feeding on nematodes. Of these, six species of *Nematoctonus*, three of *Cystopage* and two of *Stylopage* were described. Among the hyphomycetes 17 were *Dactylella* and nine each of *Arthrobotrys* and *Dactylaria.* A methodological work carried out during the period 1950–55 by Duddington (1950a,b, 1951b, 1955a) in Britain revealed several new species as well as many described by Drechsler during 1945–55 (Drehsler, 1945, 1946a,b,c, 1950a,b, 1952, 1954a,b, 1955). During the same era, Peach (1952) also described some species of the genus *Dactylaria*. Soprunov (1966) investigated soil hyphomycetes in the Soviet Union and described local

Table 3.1. Species of some predaceous fungi and their trapping mechanism.

Fungi species	Taxonomic classification	Trapping devices
Arthrobotrys brochopaga	Orbiliomycetes	Constricting rings
A. conoides	Orbiliomycetes	Adhesive networks
A. dactyloides	Orbiliomycetes	Constricting rings
A. haptotyla	Orbiliomycetes	Adhesive knobs
A. irregularis	Orbiliomycetes	Adhesive networks
A. microscaphoides	Orbiliomycetes	Adhesive networks
A. musiformis	Orbiliomycetes	Adhesive networks
A. oligospora	Orbiliomycetes	Adhesive networks
A. robusta	Orbiliomycetes	Adhesive networks
A. shizishanna	Orbiliomycetes	Adhesive networks
A. superba	Orbiliomycetes	Adhesive networks
A. thaumasia	Orbiliomycetes	Adhesive networks
Cystopage cladospora	Zygomycetes	Adhesive hyphae
Dactylaria candida	Orbiliomycetes	Adhesive knobs, non-constricting rings
D. eudermata	Orbiliomycetes	Adhesive networks
Dactylella bembicodes	Orbiliomycetes	Constricting rings
D. ellipsospora	Orbiliomycetes	Adhesive knobs
D. lobata	Orbiliomycetes	Adhesive hyphae
D. zhongdianensis	Orbiliomycetes	Adhesive networks
Dactylellina haptotyla	Orbiliomycetes	Adhesive knobs
D. sichuanensis	Orbiliomycetes	Adhesive knobs, non-constricting rings
D. varietas	Orbiliomycetes	Adhesive knobs, non-constricting rings
Drechslerella anchonia	Orbiliomycetes	Constricting rings
D. brochopaga	Orbiliomycetes	Constricting rings
D. dactyloides	Orbiliomycetes	Constricting rings
Duddingtonia flagrans	Orbiliomycetes	Adhesive networks
Geniculifera perpasta	Orbiliomycetes	Adhesive networks
Helicocephalum oligosporum	Zygomycetes	Adhesive hyphae
Monacrosporium bembicodes	Orbiliomycetes	Constricting rings
M. cionopagum	Orbiliomycetes	Adhesive networks
M. elegans	Orbiliomycetes	Adhesive networks
M. ellipsosporum	Orbiliomycetes	Adhesive knobs
M. eudermatum	Orbiliomycetes	Adhesive networks
M. gephyropagum	Orbiliomycetes	Adhesive branches
M. haptotylum	Orbiliomycetes	Adhesive knobs
M. megalosporum	Orbiliomycetes	Adhesive networks
M. psychrophilum	Orbiliomycetes	Adhesive networks
Peniophorella praetermissum	Basidiomycetes	Adhesive hyphae
Stropharia rugosoannulata	Basidiomycetes	Adhesive hyphae
Stylopage hadra	Zygomycetes	Adhesive hyphae
S. leiohypha	Zygomycetes	Adhesive hyphae

strains of the four species each of *Arthrobotrys* and *Tricothecium.* Cooke and Godfrey (1964) prepared a complete key containing 96 species of nematode-destroying fungi. Rifai and Cooke (1966) studied some didymosporus genera of nematode-trapping hyphomycetes and compared them with *Tricothecium, Geniculatia* and *Arthrobotrys*. Haard (1968) made taxonomic studies on the genus *Arthrobotrys* and reported 20 species. Barron and Davidson (1972) described *Arthrobotrys anomala* capturing its prey by adhesive nets or branches producing non-septate conidia. Schenck *et al.* (1977) reported a new nematode-trapping hyphomycete producing ameroconidia. They re-evaluated the generic concept of *Arthrobotrys* and transformed nematode-trapping species of *Dactylaria* to *Arthrobotrys.* Scheuer and

Webster (1990) described *Dactylella arcuata*, a nematophagous hyaline hyphomycete recovered from a small leaf portion of *Ammophila arenaria.* Zhang *et al.* (2005) described *Dactylella zhongdianensis*, a predaceous fungus from Zhongdian in western Yunnan, China. The fungus was characterized by its solitary clavate, multiseptate conidia borne on erect unbranched or branched conidiophores. In an another study, Zhang *et al.* (2007) collected 47 species of nematophagous fungi constituting of five genera, i.e *Arthrobotrys*, *Dactylellina*, *Drechslerella*, *Dactylella* and *Tripsporina*, from Yunnan and Guizhou province of China. *Arthrobotrys psychrophila* and *Dactylellina tentaculatum* were the two new species recorded during the investigation. These interesting fungi have also been noticed in India from Coimbatore (Sachchidananda and Rama Krishnan, 1971) and Delhi soil (Srivastava, 1986), but methodological work on isolation and morphological investigation of nematophagous fungi was undertaken by Dayal and Srivastava (1978) and some of these have been published as new records from India (Srivastava and Dayal, 1982, 1983, 1984). Askary (1996) conducted a survey on the occurrence of nematophagous fungi in two series, collecting a total of about 80 samples from various sites. In the course of this investigation, the important genera of nematode-trapping fungi encountered were *Cystopage*, *Stylopage* and *Monacrosporium* (Figs 3.1–3.3).

Ahrén *et al.* (1998) worked on the phylogeny of nematode-trapping fungi. They are of the opinion that the telemorphs of most of the nematode-trapping species are located within *Orbilia* and their taxonomic positions are arranged on the types of their trapping devices. These nematode-trapping fungi are usually described in their anamorphic stage (Liu *et al*., 2009).

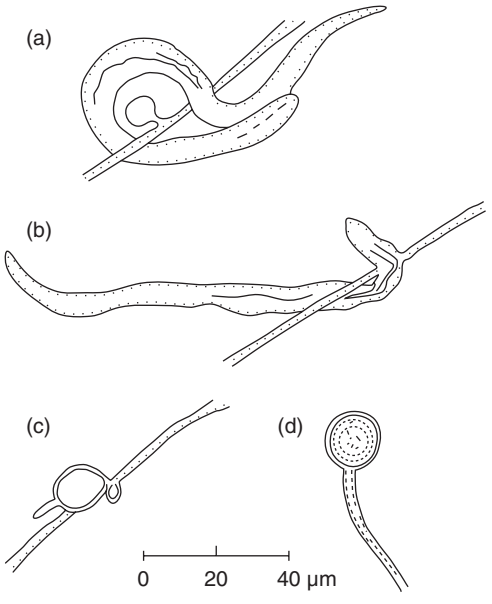

Fig. 3.1. *Cystopage cladospora*: (a) a portion of hypha with captured nematode showing young lateral chlamydospore; (b) a portion of hypha with captured nematode; (c) a portion of hypha with lateral chlamydospore; and (d) a portion of hypha with terminal chlamydospore.

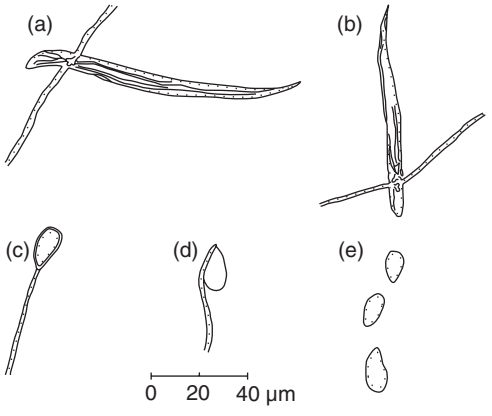

Fig. 3.2. *Stylopage leiohypha*: (a and b) a portion of hypha with captured nematode showing assimilative branch inside nematode body; (c and d) conidiophore; and (e) detached conidia showing variation in size and shape.

3.3.2 Mode of action

Zopf (1888) was the first to observe the trapping of active motile nematodes by *A. oligospora. A. oligospora* pierces the nematode cuticle with the help of a penetration tube. The nematodes that are trapped by nematode-trapping fungi struggle violently for a while and after an hour they become quiescent and die. Hyphal growth of the fungi takes place at the point where the nematode sticks to the fungi. The hypha enters into the nematode body causing a globular infection bulb at the tip. Numerous

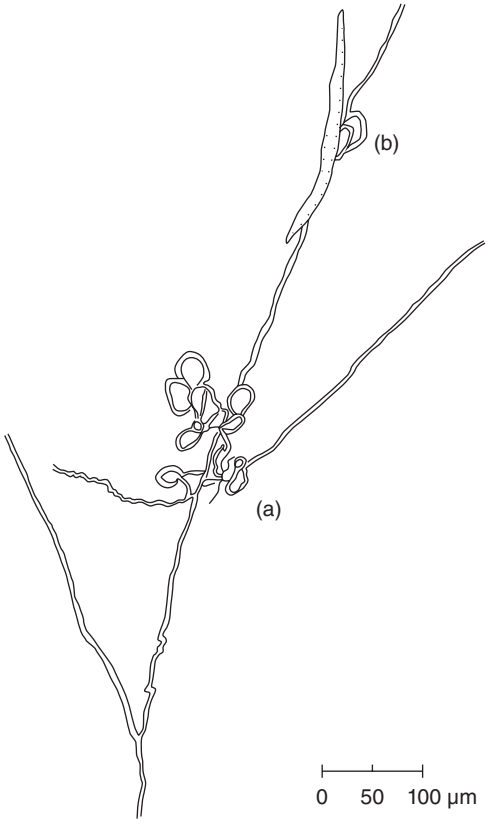

Fig. 3.3. *Monacrosporium megalosporum*: (a) a portion of mycelia hyphae producing arched or circular hyphal meshes; and (b) a portion of hypha showing entangled nematode into adhesive net.

hyphae grow out from an infection bulb, which spread inside the nematode body and absorb its contents. However, the cause of death of the nematode after its capture is still not very clear. Initially it was thought that the trapped nematode was killed either due to exhaustion or mechanical injury. Duddington (1946, 1950a) suggested the possibility of some nematode poison as cause of death. Soprunov and Galiulina (1951) reported a nematicidal substance secreted by *Arthrobotrys parvicovi* in liquid medium responsible for the death of the nematode. According to Ahman *et al.* (2002), *A. oligospora* has two pathogenicity factors: (i) carbohydrate binding protein, i.e. lectin; and (ii) extracellular serine protease, which immobilizes the trapped nematode.

It has been observed that fungi develop traps generally in the presence of nematodes. Roubaud and Descazeaux (1939) are of the opinion that stimulation for the formation of traps is due to the physical touch of the nematode with the mycelium. Lawton (1957) demonstrated that sterilized nematode extract stimulated formation of constricting rings in *Arthrobotrys dactyloides.* Soprunov (1958), on the basis of an experiment, reported that chemical stimulus is responsible for trap formation in nematode-trapping fungi. He demonstrated that nematode extract substances like liver extract, blood serum, glucose, ether, alcohol and even inorganic chemicals such as HCl, NaOH, Na_2CO_3, $CaCl_2$ and $Na(OH)_2$ stimulate trap formation. Some research workers demonstrated that a soluble substance 'nemin' is responsible for the formation of a trap structure (Pramer and Stoll, 1959; Pramer, 1964). Another opinion was that volatile metabolic products evolved by nematodes (Ellison *et al.*, 1960) might be capable of inducing nematode-trapping fungi to form traps, however, Schenck and Pramer (1975) on the basis of an experiment reported that no volatile metabolizer evolved by a nematode is capable of inducing trap formation in fungus.

Nematode-trapping fungi need carbohydrate sources to proliferate, however, factors which cause fungistasis also play a vital role in their abundant growth (Viaene *et al.*, 2006). Some fungi have the characteristics to grow more profusely in the rhizosphere and this may be due to the presence of plant-parasitic nematodes in the root-zone. *A. oligospora* have been found growing in abundance in the rhizosphere of tomato and barley plants because of its chemotropical attraction to the root tips, which provides them an opportunity to trap plant-parasitic nematodes (Bordallo *et al.*, 2002).

Nematode-trapping fungi are not obligate predators and can be grown as saprophytes in pure culture. The trapping devices are produced by these carnivorous fungi within 24 h after the nematodes are introduced. Traps can also be induced by environmental and nutritional factors. Nordbring-Hertz (1968) reported that limited nutrition is essential for trap induction, however, Balan and Lechevalier (1972) observed trap formation in some fungi when transferred from a nutrient-rich medium to a

nutrient-poor medium. The formation of traps takes place from hyphae but it has also been recorded developing from germinating conidia, particularly in the case of those fungi that produce constricting rings (Persmark and Nordbring-Hertz, 1997). Dackman and Nordbring-Hertz (1992) illustrated that trap formation by germinating conidia minimizes the resources and time required by the fungus to compete for nitrogen. In an experiment it was observed that development of trap and nematode-trapping capability of *Drechslerella stenobrocha* was increased by abscisic acid but decreased by nitric oxide (Xu *et al.*, 2011).

Adhesive three-dimensional nets are the most common type of fungal traps produced by *A. oligospora*, *Arthrobotrys superba* and *Dactylella pseudoclavata* and are constructed when the loops create a three-dimensional configuration (Moosavi and Zare, 2012). *A. oligospora* has the most common type of nematode trap. Loops are produced on loops to form a three-dimensional network. The network is coated with an adhesive containing lectin that helps to bind to the specific carbohydrate present on the nematode cuticle. *Dactylaria eudermata* produces hyphal bails and network compound to entangle the nematodes. The hyphal nets produced by this fungus may be single to two- or three-dimensional and are formed through repeated hyphal anastomosis, which captures nematodes either through the adhesive material present on the surface of hyphal nets or due to physical entanglement. Anamika and Singh (2011) in an experiment observed that inflation of hyphal bails takes 30–50 min and capturing and killing of a nematode by single conidia in water takes 35–55 h. However, real trapping and killing of a nematode in maize meal water medium (1:10) took 1 min and 30–50 h, respectively. Drechsler (1937) described the constricting ring in nematode-trapping fungi. Each ring consists of three curved cells, one of which is connected to the parent hyphae by a two-celled stalk. These rings are formed at intervals along the parent hypha and the stalk ensures that the rings are raised above and perpendicular to the fungus filament. Such a position increases the probability of a passing nematode to swim through the ring. The ring has an outer diameter of about 30 µm and aperture diameter of about 20 µm (Liu *et al.*, 2012). *Dactylaria* has a sophisticated constricting ring. Its trapping device has a short stalk with a ring made of three cells surrounding a large concentric hole. These rings are active rather than passive. The inner surface of the ring is sensitive to touch. When a nematode passes through or touches the inner surface, the volume of the three cells increases rapidly thereby reducing the size of the hole, which ultimately results in entrapping the nematode and preventing its escape (Pramer and Kuyama, 1963). After the nematode is captured, the fungus produces a penetration tube which pierces the nematode cuticle. The time taken in ring constriction is about 0.1 s and if it delays by 2–3 s the nematodes are able to escape. It appears to be a thigmotrophic response (Couch, 1937; Muller, 1958) triggered when the nematode is in contact with the inner surface of any cell of the ring. However, electron microscopy reveals that during the process of ring cell expansion, rupturing of the outer wall of the ring cells takes place along a defined line on the ring's inner surface. During the process due to release of wall pressure there is a rapid uptake of water, which causes the expansion of the elastic inner wall of the rings. Chen *et al.* (2001) studied the involvement of signal transduction pathway in the inflation of the ring cells of *A. dactyloides.* It was observed that the nematode exerts pressure on the ring, which leads to the activation of G-proteins in the ring cells. This causes an increase in cytoplasmic Ca^{++} activation of clamodium as well as opening of the water channels. The ring becomes constricted due to inner expansion of the ring cell, which immobilizes the nematode.

3.3.3 Ecology and distribution

Ecological survey on the occurrence of nematode-trapping fungi reveals that this group is present in all types of climate and habitats (Gray, 1987). Duddington (1954) reported that nematophagous fungi were common in agricultural soils, however, they were widespread and occur in a wide range of habitats. Most of the surveys have been done in temperate regions of the world and it is certain that nematode-trapping fungi have a worldwide distribution having been isolated from Canada

(Barron, 1978), England (Duddington, 1951a), New Zealand (Fowler, 1970; Wood, 1973) and the USA (Mankau and Clark, 1959), but little information is available from tropical soils. In Asian countries, some surveys have been done in India in Coimbatore (Sachchidananda and Ramakrishnan, 1971), Varanasi (Saxena and Mukherji, 1991) and Delhi (Srivastava, 1986). Barron's (1977) extensive examination of the soil of Ontario, Canada revealed that temperate soil with a long winter, dormant period contains essentially the same flora as observed in the subtropical soils of southern California (Mankau and Clark, 1959). However, survey literature indicates that information on the occurrence of nematode-trapping fungi in tropical areas is notably sparse, and the tropics may prove to be a unique area for these fungi. These fungi generally occur in the upper 20 cm of the soil but are almost absent below 40 cm (Persmark *et al*., 1996). Zhang *et al.* (2007) suggested that the horizontal distribution of freshwater nematophagous fungi is not restricted strictly by geography and climate but the distribution frequency of species differs in various habitats.

Barron (1992) is of the opinion that nematode-trapping fungi have evolved among cellulolytic or lignolytic fungi as a response to nutrient deficiencies in nitrogen-limiting habitats. In the soil ecosystem, where there is a high carbon:nitrogen ratio, nematodes might serve as an important source of nitrogen during growth on carbohydrate-containing substrates. In general, plant nematodes are parasitic on plant roots and therefore they provide an opportunity for nematophagous fungi to thrive in the rhizosphere by capturing, killing and digesting the nematodes for their nutrition. Nematophagous fungi belonging to 10–15 different species may be obtained from many soils. However, some predaceous fungi appear to be rhizosphere organisms while others occur in non-rhizosphere soil. The relationship with the plant rhizosphere may be an important aspect of the biological control capacity of these fungal species as it influences plant-parasitic nematode populations.

Cooke (1962) reviewed some of the ecological relationships of fungal predators and identified some of the problems associated with using BCAs. Before predation, mycelia growth and trap formation must occur. Both these processes require energy, which can be supplied by a readily available carbon source. Consequently the addition of organic amendments to soil is usually followed by a short period in which the activity of nematode-trapping fungi increases. In fact an increasing amount of amendment may result in a reduction in the predaceous activity of the fungus. This happens because of intensification in the activity of the soil microbes competing with the predaceous fungi for nutrients. The dual nutritional capacity of nematode-trapping fungi remains a puzzling aspect of their biology which requires further investigation to understand what initiates predaceous activity and how long it can be sustained (Mankau, 1980).

It is difficult to relate the distribution of nematode-trapping fungi to a particular environmental condition as the method of isolation is semi-quantitative. *Arthrobotrys musiformis* occurred in 50% of the soil samples at 15 cm but declined with depth, and occurrence was correlated with nematode distribution (Mankau and McKenry, 1976). Gray (1983a) studied the distribution of nematophagous fungi in Ireland and their habitat preference and compared the results with earlier surveys. Later, Gray (1984) studied the effect of soil moisture, organic matter, pH and nematode density on distribution of nematophagous fungi and their distribution on habitat relationships in soil. In 206 samples of soil, the predatory fungi were dominant in organic matter. The presence of obligate parasites was associated with high soil nematode densities while facultative predators were independent of nematode density. Al-Hazmi *et al.* (1982) conducted a greenhouse experiment to evaluate the effect of *Arthrobotrys conoides* on *Meloidogyne incognita* population densities as affected by soil temperature and inoculum density. The effect on the population densities of *M. incognita* was greater at a soil temperature of 25°C than at 18 or 32°C. *A. conoides* was most effective in nematode control when applied into the soil 2 weeks prior to nematode inoculation and planting of maize. Srivastava and Askary (2000) conducted a survey on the occurrence of nematophagous fungi from various sites of Pusa farm, Bihar, India. Two separate collections of soil samples were made, the

first during the months of February and March and the second during July to September. The collections were made on three kinds of soil habitat, i.e. poor in organic matter, rich in organic matter (decaying leaves and plant remains) and humus rich. In the course of this limited work nine fungal species representing seven genera were encountered and identified. The presence of nematophagous fungi was around 60% among the three types of soil habitat chosen. The predatory species of fungi were more frequent during July–September. Among the predators, the most common were *Monacrosporium megalosporum* followed by *Stylopage leiohypha* and *Cystopage cladospora.* Species diversity index showed maximum value in organic matter-rich soil followed by humus-rich soil. Soil poor in organic matter showed the minimum value of species diversity. In a survey conducted in South Korea in four different habitats, i.e. mountain, upland, paddy field and greenhouse, the highest incidence (95%) of nematophagous fungi was found in greenhouse and upland habitats. The most common species was *A. oligospora.* As far as trapping devices are concerned, the most abundant were net-forming species followed by constricting ring and adhesive hyphae (Kim *et al.*, 2001). Li *et al.* (2006) reported two nematode-trapping fungi, *Dactylellina sichuanensis* and *Dactylellina varietas* from China, entrapping nematodes by both adhesive knobs and non-constricting rings. Saxena (2008) reported 16 species of nematode-trapping fungi from Scotland of which *Arthrobotrys gephyropaga* and *Drechslerella brochopaga* were most common.

3.3.4 Effect on phytonematodes

Cobb (1917), the pioneer nematologist of the USA who is also considered as 'Father of American Nematology', suggested that predaceous nematodes might serve as BCAs in the management of nematodes, however, later on other eminent nematologists such as Drechsler (1941), Duddington (1962), Stirling and Mankau (1978, 1979), Kerry (1980) and Mankau (1980) summarized investigations on numerous soil microorganisms which led to a new direction in the exploitation of beneficial microorganisms for the management of plant-parasitic nematodes. Stirling *et al.* (1998), in an attempt to formulate *A. dactyloides*, observed that the fungus proliferates well from granules and consistently produces traps in soil. Under greenhouse experiments the fungus was found effective in reducing the number of root-knot nematodes in soil by up to 96%. If granules containing the fungus are applied around the roots of a transplanted crop they may substantially reduce the number of root-knot nematodes entering the roots during the first few weeks of plant growth. However, the main drawbacks of this fungus is that it is very sensitive to desiccation and therefore rapid drying procedures cannot be used and a diphasic system (transfer to a solid substrate after liquid culture to complete mass production) may not be commercially viable. Hams and Wilkins (1961) reported successful control of root-knot nematode in pea with lyophilized liquid medium cultures of *Arthrobotrys robusta* and *Dactylaria candida.* In a laboratory experiment it was found that adults and juveniles of *Pratylenchus penetrans* are killed when added to the fungus culture of *A. dactyloides*, *A. oligospora*, *Monacrosporium ellipsosporum* and *Monacrosporium cionopagum* (Timper and Brodie, 1993). In India, research has been carried out in this field by some noted workers, such as Sachchidananda and Rama Krishnan (1971) in Coimbatore soil, where *Stylopage hadra* was found active in destroying nematode species of *Cephalobolus* whereas *Dactylella asthenophaga* was active in capturing and killing root-knot nematode, *Meloidogyne arenaria.* Dayal and Srivastava (1978) found *Arthrobotrys arthrobotryoides* and *Arthrobotrys anchonia* trapping and killing the species of *Rhabditis* in Varanasi soil. Later on in 1982, Srivastava and Dayal reported *M. megalosporum* and *M. salinum* capturing and killing many species of nematodes in agriculture soil. Townshend *et al.* (1989) coated tomato seeds with *Meria coniospora* and planted them in *Meloidogyne hapla*-infested soil. After 28 days, a reduction of 34% in galling and 47% in the population of J_2 was observed. When the roots of tomato transplants were dusted with *M. coniospora* fungus rye-powder or sprayed with a spore suspension before planting in root-knot nematode, *M. hapla*-infested soil, the reduction in root galling was 42 and 35%, respectively, in 28 days;

however, there was no reduction in soil population of J_2 and improvement in plant growth. Duponnois *et al.* (1997) tested the trapping ability of *A. oligospora*, *A. conoides*, *Arthrobotrys* sp., *Dactylaria shelensis* and *Dactylaria* sp. against the root-knot nematode, *Meloidogyne mayaguensis.* Most of the *Arthrobotrys* strains and one *Dactylaria* strain decreased the development of the nematodes. *Dactylaria brochopaga* isolated from Egyptian soils have been found more effective in capturing the nematode prey, dissolving its outer cuticle and digesting the inner content (Aboul-Eid, 1963; Aboul-Eid *et al.*, 1997, 2002). It has been demonstrated that the efficacy of various fungi differs in trapping and parasitizing nematodes. *A. dactyloides* was found more efficient than *Dactylella brochopaga* and *Monacrosporium eudermatum* in trapping the nematode *Meloidogyne graminicola.* Biro-Stingli and Toth (2011) evaluated *A. oligospora* against root-knot nematode, *M. hapla* infesting green pepper in Hungary. The results indicated a 35% reduction in the female population of the nematode. Zouhar *et al.* (2010) evaluated six strains of nematopathogenic fungi, i.e. *A. oligospora* (strain CBS 115.81), *Dactylella oviparasitica* (strain CBS c347.85), *Dactylellina candida* (strain CBS 546.63), *Dactylellina lysipaga* (strain CBS 581.91), *Dactylellina phymatopaga* (strain CBS 450.93) and *P. chlamydosporia* (strain CBS 113566), against three species of plant-parasitic nematodes, i.e. *Ditylenchus dipsaci*, *Globodera rostochiensis* and *M. hapla. A. oligospora* proved the most pathogenic fungus to all three tested species of nematodes. In another experiment, five isolates of *A. oligospora* isolated from different parts of India were evaluated against *M. incognita* and *Rhizoctonia solani* on tomato plants grown under greenhouse and field conditions. *A. oligospora* strain VNS-1 offered significant disease reduction in terms of galls and seedling mortality. It was concluded that *A. oligospora* can be a better environment-friendly option and can be incorporated in the integrated disease management module of crop protection (Singh, U.B. *et al.*, 2013).

3.3.5 Formulation and commercialization

A French company produced Royal 300®, based on nematode-trapping fungi *A. robusta* var. *antipolis* for the control of *Ditylenchus myceliophagus* in mushroom (Cayrol *et al.*, 1978) and Royal 350®, a formulation of *Arthrobotrys irregularis* for the control of *Meloidogyne* in tomato (Cayrol and Frankowski, 1979). These results have provided some encouraging impetus among workers in this field (Cayrol *et al.*, 1978; Cayrol, 1981). Granular formulations of *Dactylella candida* and *A. dactyloides* were prepared by Stirling and Mani (1995) by encapsulating different quantities of fungal biomass in alginate or by subjecting encapsulated biomass to further fermentation. The formulations thus prepared showed that the presence of nutrients and the quality and quantity of biomass in granules determine the level of activity against nematodes. Noweer and Aboul-Eid (2013) reported that *Dactylaria brochopaga* alone or in combination with yeast, molasses or vermiculite as a carrier reduced the population of root-knot nematode *M. incognita* in soil and galling on the roots of cucumber.

3.4 Endoparasitic Fungi

Endoparasitic fungi are obligate parasites of nematodes that spend their entire vegetative lives inside the nematode they infect. Nematodes may encounter the spores (conidia or zoospores) while moving through the soil pores. The spores infect the nematode in two ways: (i) pre-oral, i.e. through the mouth when the spores are ingested by the nematodes together with food; or (ii) percutaneous, i.e. spores adhere to the nematode's cuticle (Table 3.2). In this case the zoospores swim towards the nematode and encyst around the natural orifices such as mouth, anus or vulva. The vegetative stage of the fungus starts with the penetration of the host body by the encysted zoospores. In the advance stage, formation of sporangium takes place with the hypha.

3.4.1 Taxonomy and morphology

All the fungi that utilize zoospores (motile spores that are propelled by one or two flagella) to parasitize their prey are placed in the

Table 3.2. Species of some endoparasitic fungi and their mode of infection.

Fungi species	Taxonomic classification	Mode of infection
Catenaria anguillulae	Chytridiomycetes	Zoospores
C. vermiformis	Chytridiomycetes	Zoospores
Chlamydomyzium anomalum	Oomycetes	Zoospores
C. sphaericum	Oomycetes	Zoospores
Drechmeria coniospora	Deuteromycetes	Adhesive conidia
Haptocillium bactrosporum	Sordariomycetes	Adhesive conidia
H. balanoides	Sordariomycetes	Adhesive conidia
H. obovatum	Sordariomycetes	Adhesive conidia
Haptoglossa dickii	Oomycetes	'Gun cells', injection
H. erumpens	Oomycetes	'Gun cells', injection
H. heteromorpha	Oomycetes	'Gun cells', injection
H. heterospora	Oomycetes	'Gun cells', injection
H. mirabilis	Oomycetes	'Gun cells', injection
H. zoospora	Oomycetes	'Gun cells', injection
Harposporium anguillulae	Deuteromycetes	Ingested spores
H. bysmatosporum	Deuteromycetes	Ingested spores
H. leptospira	Deuteromycetes	Ingested spores
Hirsutella rhossiliensis	Deuteromycetes	Adhesive spores
Gonimochaete horridula	Oomycetes	Adhesive spores
G. latitubus	Oomycetes	Adhesive spores
G. lignicola	Oomycetes	Adhesive spores
G. pyriforme	Oomycetes	Adhesive spores
Meria coniospora	Deuteromycetes	Adhesive conidia
Meristacrum asterospermum	Zygomycetes	Adhesive conidia
Myzocytiopsis glutinospora	Oomycetes	Zoospores
M. humicola	Oomycetes	Zoospores
M. intermedia	Oomycetes	Zoospores
M. lenticularis	Oomycetes	Zoospores
M. papillata	Oomycetes	Zoospores
M. zoophthora	Oomycetes	Zoospores
Nematoctonus concurrens	Basidiomycetes	Adhesive hour-glass knobs, adhesive spores
N. leiosporus	Basidiomycetes	Adhesive hour-glass knobs, adhesive spores
Olpidium vermicola	Chytridiomycetes	Zoospores
Pythium (Lagenidium) caudatum	Oomycetes	Zoospores
Spirogyromyces vermicola	Unknown	Ingested spores
Verticillium balanoides	Deuteromycetes	Adhesive spores

division Mastigomycotina. Out of five zoospore-producing fungi which are found in this division, three belong to nematophagous species (Esser and Schubert, 1983). The posteriorly uniflagellate fungi (chytrids) have been placed into three phyla, i.e. Blastocladiomycota, Chytridiomycota and Neocallimastigomycota (James *et al.*, 2000, 2006; Letcher *et al.*, 2006; Hibbett *et al.*, 2007). Hodge *et al*. (1997) reported two nematode-parasitic isolates of *Harposporium* forming synanamorph attributed to the *Hirsutella.* One was attributed as *H. anguillulae* whereas the other was described as a new species *H. cerberi. Acrostalagmus bactrosporus* and *Acrostalagmus obovatus*, the two endoparasitic fungi that infect by spore adhesion, have been transferred to the genus *Haptocillium* (Glockling and Holbrook, 2005). Guima and Cooke (1972) described a new species *Nematoctonus tripolitanius* and also gave the key to the known species of *Nematoctonus.* Saikawa and Arai (1986) revealed the adhesive secretion in *N. pachysporus* surrounding the knob of the conidial outgrowth to be an irregular mass of

randomly oriented fibrillose materials. The fibrillose mass becomes attached to the nematode prior to hyphal penetration. In *Haptoglossa zoospora*, the contents of the infective thallus cleave to form primary spores. The encysted spores are homologous with the spherical primary spores of *Haptoglossa heterospora.* The peculiar shape of secondary glossoid spores relates to a unique mechanism of infection whereby infective tertiary spores infect the nematode in an extremely short period of time. Drechsler (1968) observed parasite-destroying nematodes from west Maryland and named them *Harposporium cycloides.* Saikawa and Morikawa (1985) studied initiation by conidia of *Harposporium subliforme.* Barron (1973) described a few nematophagous fungi which were new endoparasites and later on in 1976 and 1977 he described six species of *Myzocytium* and one species each of *Lagenidium* and *Protoascus* (Barron, 1976, 1977).

3.4.2 Mode of infection

Endoparasitic nematodes use their spores to infect nematodes, which adhere on the nematode's cuticle or are ingested together with food. After locating a host, zoospores become attached to the nematode cuticle, often near a natural opening such as mouth, anus or vulva, shed their flagellum, encyst it and become sedentary. Infection of the nematode integument by encysted zoospores takes place with the formation of a penetration tube that enters through an orifice or penetrates the nematode integument directly. Electron microscopy reveals that the spores adhered to the nematode's cuticle develop a swelling at the point of contact and the penetrating germ tube which grows through the cuticle is filled with dense vesicles containing digestive enzymes (Glockling and Beakes, 2000). Saikawa and Anazawa (1985) showed the narrow germ tube of *Gonimochaete* growing from the apical knob and penetrating the nematode cuticle after adhesion. Zoospores are produced in a flask-shaped zoosporangium either in or out of the body of the host. Zoosporangia produce a short or very long evacuation tube and from the mouth of the evacuation tube emerges the zoospores that swim to their prey, attach to its integument and penetrate it by an infection peg. Zoospores of *Pythium caudatum* are attracted to secretions from the body orifices of the nematode and encyst in clusters on these locations, germinate and produce hyphae that pass through the body orifice into the nematode. The enzymes secreted by the fungi help in digesting the body contents of the host. Deacon and Saxena (1997) reported that the spores of *Catenaria anguillulae* are attracted to the orifices of the nematode and express consistent orientation of encystment and cyst germination. Zoospores of *C. anguillulae* attach in clusters on the natural body opening of the nematode. Zoospores of *Catenaria auxiliaris* infect immature female cyst nematodes when they emerge from the root, thus stopping egg formation. It is not clear how the zoospores of endoparasitic fungi locate their host. Glockling and Beakes (2000) are of the opinion that zoospores follow chemostatic stimuli that emanate from the nematode secretions and that is why zoospores of some species encyst in large numbers at the nematode orifices. *Nematopthora gynophila* destroys the internal tissues of the cuticle body wall as well as eggs. The infection caused by *N. gynophila* prevents the development of a cyst, the body loses its turgor and the cyst is filled with spores within 4 days. The spores of these fungi remain viable in soil for at least 5 years. Fungi belonging to the genus *Harposporium* produce spores of different shapes. A nematode moving in the soil ingests the spores that become stuck in its oesophagus and the infection in the nematode begins with the germination of the spore. The conidiophores of *H. anguillulae* have crescent-like curvature and pointed tips resembling a fishhook, which fix in the oesophagus of nematodes. The conidiophores of *H. anguillulae* enter the nematode host by the oral route and it is assumed that this may be due to swallowing of conidiophores by nematodes (Aschner and Kohn, 1958). Some endoparasitic fungi are host-specific and their spores adhere to and infect only a few species of nematode. Askary (1996) in an investigation observed that *Harposporium arcuatum* have a strong nematode-attracting mycelia although the conidia were not attracting. The fertile hyphae produced from the nematode cadaver attracted other nematodes to the

vicinity of the conidia, which was then ingested by some bacterial feeding nematodes together with their food. The conidia lodged in the oesophagus of the nematode and eventually started germination (Figs 3.4, 3.5). In general the bacterial feeders were attacked and few plant-parasitic nematodes were the victim of *Harposporium.* In contrast, *Gonimochaete* were more aggressive and have a wide host range, infecting bacterial feeding, fungal feeding and plant-parasitic nematodes.

Some endoparasites such as *Drechmeria coniospora* and *Hirsutella rhossiliensis* develop an adhesive bud on their conidia with which they infect the nematode. *Nematoctonus*, the name of which means 'nematode murderer', spend their entire vegetative stages inside the nematode. It captures the nematode with both adhesive traps and adhesive spores, thus constituting a link between the two groups. *N. leiosporus* have special traps known as hour-glass traps which are formed on the mycelim in the presence of nematodes. Around the tip of the side branch of hour-glass traps; a special blob of mucilage is secreted, which becomes attached to the nematode cuticle (Webster *et al.*, 1995). In the case of *Nematoctonus concurrens*, the conidia adhere to the cuticle of the nematode. The killing of the nematode is caused by a toxin. The fungus penetrates the nematode's body and digests its contents. *Hirsutella rhossiliensis* produces non-motile spores that stick to the nematode's body passing through the soil, penetrate the cuticle and cause infection. Germination of spores takes place inside the body of the nematode and within a few days the nematode is killed and its entire body cavity is filled with hyphae. Sporulation of fungus takes place from the dead nematode; spores are borne on bottle-shaped phialides on hyphae that radiate from the cadaver into the soil.

The mechanism of infection by gun cells was demonstrated and illustrated for the first time in *Haptoglossa mirabilis* (Robb and Barron, 1982; Robb and Lee, 1986a,b). Beakes and Glockling (1998) made a comprehensive study of this topic, taking the case of *Haptoglossa dickii.* Here, the needle chamber is a broadened walled region of the tube that contains a needle-like penetration missile surrounded by several overlying cones. These cones function to keep the needle in place. The spores are kept under pressure by a large basal vacuole. It is stimulated to fire by contact with a passing nematode, due to which the tube everts explosively, firing the needle through the nematode cuticle to insert the lower portion of the tube into the body of the nematode. The tube tail is un-walled and as soon as the organelles rush into it, it expands to form a sporidium. The gun cell can only fire once. In the case of *H. dickii*, it has been observed that the firing mechanism does not respond readily to artificial stimuli (Beakes and Glockling, 1998), whereas in *H. mirabilis* the gun cells can be induced to fire by pressure (Barron, 1980, 1987).

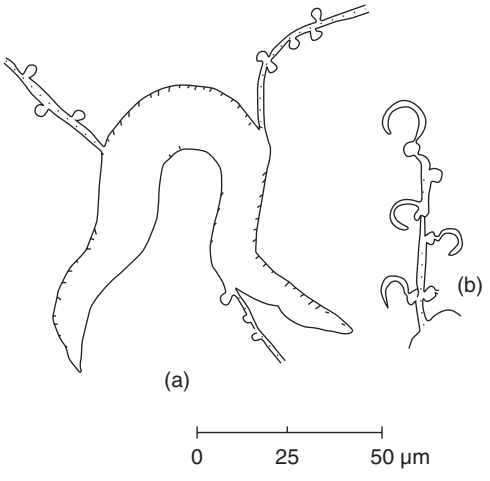

Fig. 3.4. *Harposporium arcuatum*: (a) conidiophorus branch and phialides developing from infected nematode cadaver; and (b) variation in the production and form of phialides and conidia.

3.4.3 Ecology and distribution

Endoparasitic fungi are reported from almost all parts of the world such as Central America (Persmark *et al.*, 1996), the USA, Canada (Gams and Zare, 2003), Great Britain (Minter and Brady, 1980), New Zealand (Hay, 1995), Germany (Kuhn, 1877), Ireland (Gray, 1983a), China (Chen *et al.*, 2000; Xiang *et al.*, 2009), India (Singh, 1967; Srivastava and Askary, 2000), El Salvador (Bucaro, 1974) and even in the soil of maritime Antarctica (Gray, 1982, 1984; Gray *et al.*, 1982).

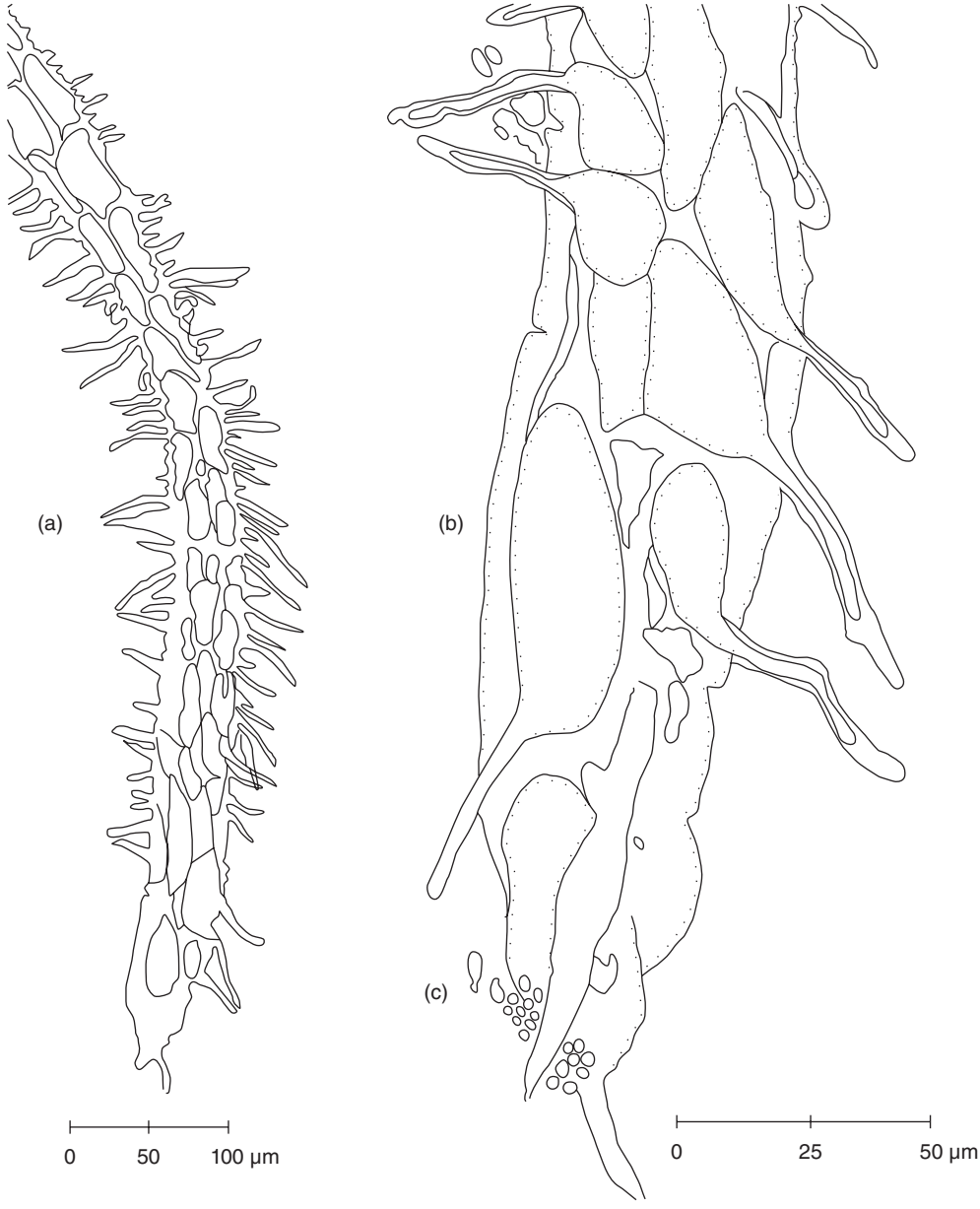

Fig. 3.5. *Gonimochaete pyriforme*: (a) a view of typical infected nematode with aerial evacuation tube; (b) a portion of typical infected nematode with aerial evacuation tube; and (c) spores having rounded off their angular outline and developing as adhesive knob at the distal end.

Catenaria auxiliaris has been reported to occur in North Europe, the USA and Australia (Kerry and Crump, 1977). Singh (1967) reported *Catenaria vermicola* from India. Gray (1983b, 1984) carried out quantitative studies on the occurrence of endoparasitic fungi and showed that temporary agricultural pasture, coastal vegetation and coniferous leaf litter had the greatest percentage of these fungi. Srivastava and Askary (2000) reported that endoparasitic nematodes

such as *C. vermicola*, *C. anguillulae*, *H. arcuatum*, *Gonimochaetae pyriforme*, *H. zoospora* and *H. heterospora* are mostly favoured by humus-rich soil and during the rainy season when the soil has sufficient moisture. Under laboratory conditions these fungal BCAs were found efficiently parasitizing different plant-parasitic nematode species (Figs 3.6–3.8).

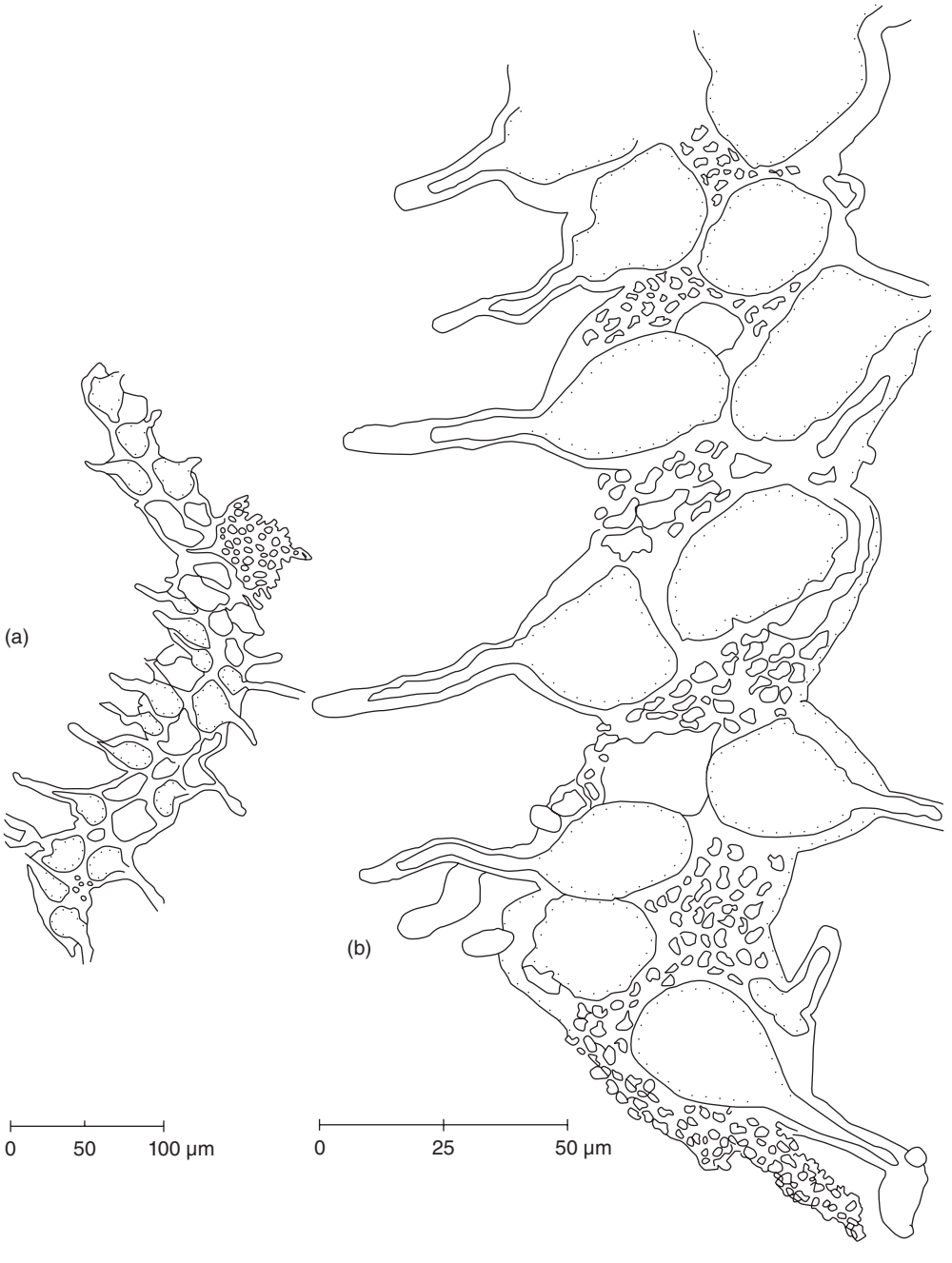

Fig. 3.6. A nematode cadaver infected with *Catenaria anguillulae*: (a) double chain of mature and immature sporangium (low magnification); and (b) a portion of double chain of mature and immature sporangium (high magnification).

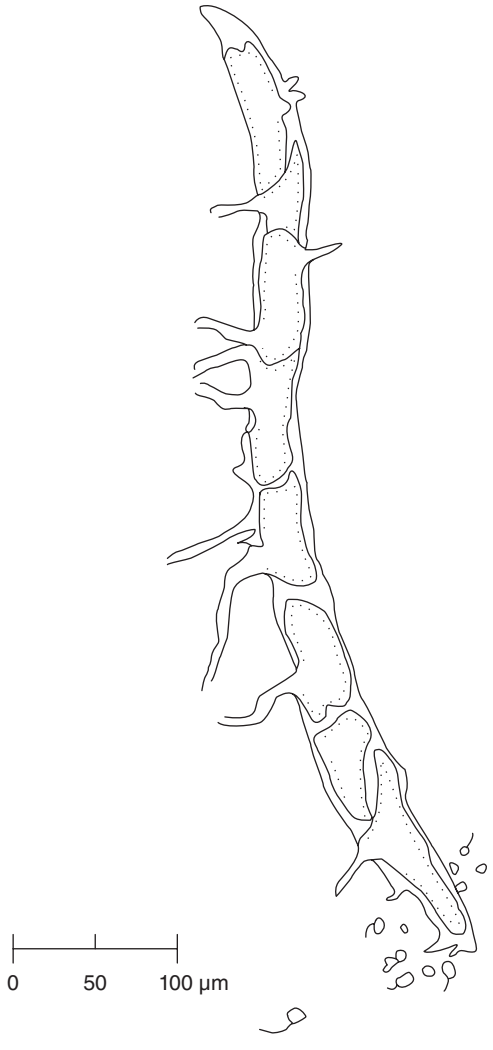

Fig. 3.7. A nematode cadaver infected with *Catenaria vermicola*. Chain of mature and immature sporangium showing a release of zoospores.

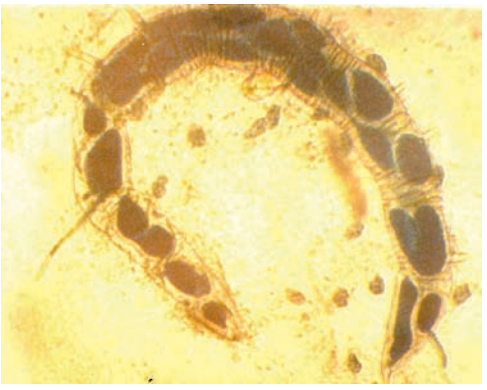

Fig. 3.8. A dead and consumed nematode with mature and immature thalli of *Haptoglossa heterospora.*

Chen and Liu (2007) studied the effect of tillage on parasitism of second stage juveniles (J_2) of soybean cyst nematode *Heterodera glycines* by endoparasitic fungi *H. rhossiliensis* and/or *Hirsutella minnesotensis* on soybean and maize. Highest percentange of juveniles parasitized by the fungus was in soybean fields compared to maize fields. The parasitization of J_2 was more in mid-season than at planting and harvest time.

Environmental factors have a great impact on the activity of endoparasitic fungi. Xiang *et al.* (2009) studied the effect of environment on the abundance and activity of *H. minnesotensis* against soybean cyst nematode, *H. glycines.* Soil temperature, soil texture and soil moisture significantly affected the activity of *H. minnesotensis* and the maximum parasitization of J_2 was observed at 10 and 15°C and 6–10% soil water contents. A lower soil temperature accompanied with lower water content and higher fine particle content resulted in greater potentiality of *H. minnesotensis* to multiply and infest nematodes.

Wet soil is the requirement of zoospore-forming fungi because movement of the zoospore is favoured by large, water-filled soil pores. Gupta *et al.* (2004) studied effects of different factors on virulence of the endoparasitic fungus *C. anguillulae.* The susceptibility of the ring nematode, *Criconemella xenoplax* to invasion by the fungus increased when exposed to 40°C for 2 h (Jaffee and Zehr, 1982). Gupta *et al.* (2003) compared the nutritional value of different oil cakes as media to culture *C. anguillulae.* Dijksterhuis *et al.* (1993) showed that conidia of *D. coniospora* were able to adhere to the nematode *Acrobeloides buetschilii.* The spores adhered to 70% of the nematodes for 16 h but barely infected the nematodes. In the Antarctic ecosystem, endoparasitic fungi with adhesive and nematode-attracting conidia were found more abundant and active over those whose conidia were ingested before infection could occur (Gray and Smith, 1984). Gray (1988) evaluated the effects of major soil nutrients and seven

common metals on the distribution of nematophagous fungi by comparing the concentration of the elements in soils with and without nematophagous fungi. It was noted that concentrations of heavy metals were able to restrict the distribution of fungi; however, *M. coniospora* was found tolerant to all metals and to copper in particular. Jaffee and Zehr (1983) reported that certain cations act to enhance parasitism, while the sulfate anion appears to inhibit parasitism. They are of the opinion that certain constituents of the soil solution may have some important effect upon the activity of parasitic soil microorganisms and, therefore, there is a need for thorough understanding of the behaviour and biology of both host and parasite so that the parasitic interaction may be enhanced to achieve a good result.

3.4.4 Effect on phytonematodes

Endoparasitic fungi have little potential in plant-parasitic nematode control because the spores of these fungi are not ingested by stylet-bearing nematodes (Zehr, 1985), however, the spores adhering to the body are helpful in checking the nematode population in soil. Kuhn (1877) was the first to report parasitization of female cyst nematodes by a fungus in Germany. The fungus identified was *Tarichium auxiliare* found in the sugarbeet cyst nematode, *H. schachtii.* Later on *T. auxiliare* was re-described as *C. auxiliaris.* Chowdhry and Dhawan (1984) reported that *C. vermicola* destroys the eggs and larvae of cereal cyst nematode *H. avenae.* Pathogenicity of *H. minnesotensis* against soybean cyst nematode has been well proven (Chen *et al.*, 2000). A reduction in the population of *M. hapla* by 61–98% in tomato roots was recorded with the application of *H. minnesotensis* (Mennan *et al.*, 2006). Combined application of *H. minnesotensis* and N-Viro Soil® (NVS), a recycled municipal biosolid, resulted in greater suppression of the population of *M. hapla* in tomato than either of the treatments alone (Mennan *et al.*, 2007). Marziano *et al.* (1995) reported a strong parasitic aptitude of *H. rhossiliensis* against cyst nematode *Heterodera daverti.* Del Sorbo *et al.* (2003) conducted a survey in Naples, Italy and reported high incidence of parasitism of *H. rhossiliensis* on the juveniles of cyst nematode *H. daverti.* Kerry (1979) reported parasitization of females by a fungus on the root surface, causing breakdown of the nematode cuticle and preventing cyst formation of cereal cyst nematode *H. avenae.* Jaffee and Zehr (1982) found *H. rhossiliensis* to be widespread in South Carolina peach orchard soils, infecting *C. xenoplax. In vitro* production of hyphae of *H. rhossiliensis* and its addition to the soil without organic substrate has been found effective as a BCA against sugarbeet cyst nematode *Heterodera schachtii* (Lackey *et al.*, 1992). However, a demerit of this fungus is that the detached spores from the phialides are unable to adhere to the body of the nematode and therefore the fungus cannot be successfully established merely by addition of spores in soil. *H. rhossiliensis* is an obligate parasite, therefore unless supplied with a minimal nematode density its local population will go extinct in soil. Hence, for successful establishment of this fungus in soil, the plant-parasitic nematode population should be above threshold level. Hay and Bateson (1997) tested the efficacy of *H. rhossiliensis* and *Verticillium balanoides* in the management of the plant-parasitic foliar feeding nematode *D. dipsaci* infesting white clover. *V. balanoides* was found superior to *H. rhossiliensis* as a BCA of *D. dipsaci.*

3.4.5 Formulation and commercialization

Though endoparasitic fungi are promising in the management of plant-parasitic nematodes, no commercial product from this group of fungi has yet been available in the market. However, formulations of *H. rhossiliensis* as alginate pellets have been reported to be used against nematodes in laboratory and greenhouse conditions (Lackey *et al.*, 1993; Jaffee *et al.*, 1996).

3.5 Egg- and Female-parasitic Fungi

Egg- and female-parasitic fungi are those which use appressoria or zoospores to infect the eggs and sedentary females of plant-parasitic nematodes (Lopez-Llorca *et al.*, 2008; Table 3.3).

Table 3.3. Species of some nematode egg-and female-parasitic fungi and their infection mechanism.

Fungi species	Taxonomic classification	Mode of infection
Dactylella ovaparasitica	Orbiliomycetes	Appressoria
Helicocephalum oligosporum	Zygomycetes	Adhesive hyphae
Lecanicillium psalliotae	Deuteromycetes	Appressoria
Nematophthora gynophila	Oomycetes	Zoospores
Olpidium vermicola	Chytridiomycetes	Zoospores
Paecilomyces lilacinus	Deuteromycetes	Appressoria
Pochonia chlamydosporia	Deuteromycetes	Appressoria
P. rubescens	Deuteromycetes	Appressoria
Rhopalomyces elegans	Zygomycetes	Appressoria

Endoparasitic nematodes such as *Heterodera* spp., *Globodera* spp. and *Meloidogyne* spp. spend most of their life cycle within the plant roots (Hussey and Grundler, 1998) and therefore are difficult to manage. Chemical management of these nematodes is effective but not sustainable in the long term. An alternative to this control measure is the fungal antagonists which are egg-and female-parasitic. This group of fungi can survive saprotrophically in the rhizosphere and are relatively easy to mass culture (Moosavi and Zare, 2012). An additional advantage of their potentiality is that their hosts are generally sessile in the form of eggs, developing juveniles and sedentary females. Several fungi, e.g. *Dactylella ovaparasitica*, *P. lilacinus*, *N. gynophila*, *A. niger*, *P. chlamydosporia*, *Olpidium vermicola*, *T. harzianum* and *Lecanicillium psalliotae*, have been reported to parasitize eggs and females of sedentary endoparasitic nematodes. However, in the last two decades four among these, *P. lilacinus*, *T. harzianum*, *P. chlamydosporia* and *A. niger*, have been exploited successfully in a commercial way for the management of endoparasitic nematodes, particularly root-knot and cyst nematodes. Keeping in view the practical significance of these four fungal BCAs, they have been described separately below.

3.5.1 *Paecilomyces lilacinus* (= *Penicillium lilacinum*) (Thom, 1910) (Thom) Samson, 1974

Paecilomyces lilacinus, a facultative fungal parasite, is an effective BCA of root-knot, reniform and cyst nematodes. The fungus sometimes infects nematodes in their mobile stages, however, it is most aggressive against nematode eggs (Onions *et al.*, 1981; Jacobs, 2002). Lysek (1976) was the first to observe its association with nematode eggs. Later on it was reported from Peru, parasitizing the eggs of *M. incognita* and *Globodera pallida* (Jatala *et al.*, 1979). The biocontrol potential of this fungus has been found equivalent to any commonly used nematicide. Its field application has been found efficient in controlling *M. incognita* (Cabanillas *et al.*, 1989a; Mittal *et al.*, 1995). Under field and greenhouse experiments, *P. lilacinus* has been reported to limit the population of root-knot nematodes, root galling and increasing the yields of plants (Godoy *et al.*, 1982; Dube and Smart, 1987; Hewlett *et al.*, 1988). Hawaii state quarantine branch has listed *P. lilacinus* among the non-restricted microorganisms (Schenck, 2004).

Taxonomy and morphology

TAXONOMIC POSITION

Division: Eumycota
Class: Deuteromycetes
Order: Moniliales
Family: Moniliaceae
Genus: *Paecilomyces*

MORPHOLOGY *Paecilomyces lilacinus*, a lilac to purple coloured soil hyphomycete discovered by Jatala *et al.* (1979) is considered as one of the most promising and practicable BCA for the management of plant-parasitic nematodes (Morgan-Jones *et al.*, 1984; Jatala, 1985). *P. lilacinus* shows fast hyphal growth and forms a dense mycelium which gives rise to conidiophores. Conidiophores are ramified in grouped branches or irregular. The conidiophores bear

bottle- shaped phialides having a swollen basal part which tapers into a thin distinct neck from the ends of which spores are formed in long chains (Jatala, 1986). Conidia are lilac in colour, ellipsoid, 2.5–3.0 µm long and 2.0–2.2 µm broad (Brand *et al*., 2010). Chlamydospores are absent (Samson, 1974).

Mode of action

Adult females of sedentary nematodes are infected when *P. lilacinus* enters its hyphae into the vulva and anus of the nematode (Jatala *et al*., 1979; Fig. 3.9). The fungus penetrates the egg and develops profusely inside and over the eggs, completely inhibiting juvenile development (Khan *et al*., 2006; Nasr Esfahani and Ansari Pour, 2006). The hyphal tips swell to form an appressorium on the egg surface. Below the appressorium is found a penetration peg, which grows into the eggshell. The infected eggs swell and buckle and, as penetration continues, the vitelline layer of the egg splits into three bands and a large number of vacuoles. The lipid layer disappears at this stage (Morgan-Jones and Rodriguez-Kabana, 1988). After penetrating the egg, hyphae grow rapidly and destroy the juvenile within it. Later on, a large number of conidiophores are produced and the hypha moves towards adjacent eggs. Adhesion between the appressorium and nematode egg surface must be strong enough to withstand the opposing force produced by the extending tip of a penetrating hypha (Money, 1998).

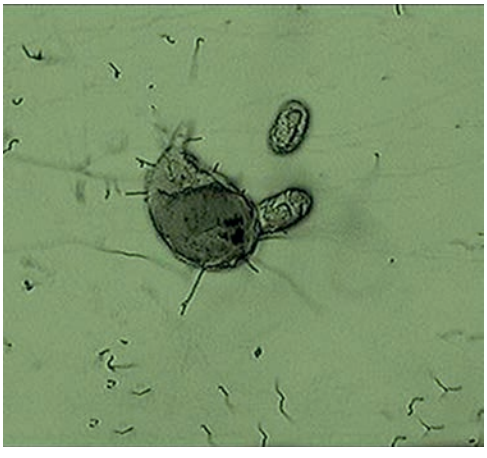

Fig. 3.9. Adult female and eggs of *Tylenchulus semipenetrans* parasitized by *Paecilomyces lilacinus* (courtesy of P.R.P. Martinelli).

Paecilomyces lilacinus has been reported to produce antibiotics, i.e. leucinostatin and lilacin (Morgan-Jones and Rodriguez-Kabana, 1988) and enzymes such as protease and chitinase. The enzyme serine protease has nematicidal activity (Bonants *et al*., 1995; Sharma and Pandey, 2009), causes degradation of the eggshell and inhibits hatching (Zareen *et al*., 2001a), whereas chitinase breaks down the eggshell thereby making the route for the fungus to pass through. The action of chitin against nematodes may be explained in two ways: (i) decomposition of chitin releases ammonia, which has a nematicidal activity on the J_2 of root-knot nematode; and (ii) chitin may increase population of chitinolytic microbiota, which parasitize eggs and egg sacs of the nematode (Mittal *et al*., 1995).

Ecology and distribution

As evidenced by the data obtained from different countries, *P. lilacinus* adapts well in varied climatic conditions (Jatala, 1986; Holland *et al*., 2001) but is most frequent in warmer regions (Domsch *et al*., 1980). This fungus is cosmopolitan and can be found in a wide range of habitats including cultivated and uncultivated soils, forests, grasslands and even deserts. In sandy soil, its distribution is limited up to a depth of 15 cm from the upper soil surface (Hewlett *et al*., 1988). *P. lilacinus* is a good root colonizer (Cabanillas *et al*., 1988) and can grow over a wide range of temperature and pH on different plant and animal substrates (Samson, 1974; Jatala, 1986; Alam, 1990; Domsch *et al*., 1995). Kiewnick and Sikora (2006) evaluated *P. lilacinus* strain 251 under different temperature regimes against *M. hapla*. The results showed a significant interaction between the nematode inoculum density and fungal treatment at low temperature at which few root-knot galls and egg masses were formed. At favourable temperature (25°C) the efficacy of the fungal parasite reached up to 90%. Maximum growth of fungi as determined by dry weight of the mycelium was observed at 24–30°C. Cabanillas *et al*. (1989a) reported that

the fungus was highly effective in controlling root-knot nematodes at an optimum temperature between 16 and 28°C. In order to enhance the distribution of this fungal antagonist, International Meloidogyne Project (IMP) sent its culture to the research workers working in 46 countries of the world (Jatala, 1985).

Effect on phytonematodes

The association of *P. lilacinus* with nematode eggs was for the first time observed by Lysek (1966). Later, Jatala *et al.* (1979) reported the fungus parasitizing eggs of *M. incognita* in Peru. The fungus has also been isolated from cyst-forming nematodes, root-knot nematodes and soil in many locations (Stirling, 1991; Stirling and West, 1991). *P. lilacinus* is sometimes also able to infect mobile nematode stages or sedentary females, but are mostly aggressive against nematode eggs (Cabanillas and Barker, 1989). Evaluation of *P. lilacinus* on potted plants and field plots have shown that the fungus has the efficacy to control a range of nematode species on a number of crops worldwide (Jatala, 1985; Alamgir Khan *et al.*, 1997). Walters and Barker (1994) studied the effect of rice-cultured *P. lilacinus* on reniform nematode, *Rotylenchulus reniformis* infesting tomato cv. Rutgers. The fungus suppressed the nematode population and increased shoot and fruit growth in both greenhouse and field conditions. In pots, pigeonpea seeds treated with *P. lilacinus* at 2×10^8 spores/kg seed against *Meloidogyne javanica* resulted in a significant reduction in galling and egg mass production on pigeonpea roots (Askary, 2012). Simon and Pandey (2010) applied *P. lilacinus* at 4 g/kg soil against *M. incognita* in pots in which okra seeds were sown. The results obtained after 90 days revealed a significant reduction in root galls as compared to control.

PI-181, a strain of *P. lilacinus* isolated from root-knot nematode infecting tomato, was grown on starch-rich grains and tested with different natural and synthetic inert carrier materials. The best emerged parameter in terms of colony count, shelf life, easy dispersibility and suppressing the nematode population was attapulgite-based clay dust powder + peat powder + acacia gum powder (Pandey *et al.*, 2011a). In a laboratory experiment three isolates of *P. lilacinus* (PlA#1, PlA#2 and PlK#01) were investigated for their enzymatic activities on different solid media. All the strains of *P. lilacinus* exhibited polysacchrolytic, proteolytic and lipolytic activities on various specific agar medium. Crude culture extracts obtained from liquid culture of *P. lilacinus* strains were incubated for 7 days, 14 days and 21 days. The mortality test of *M. incognita* (J_2) was done by these extracts after incubation for 24, 48 and 72 h. All the strains used in the experiment exhibited a larvicidal effect against the nematodes. Highest percentage mortality was caused by *P. lilacinus* strain PIA#1, whereas strain PIK#01 exhibited the lowest percentage of mortality after 72 h corresponding to incubation of 14 days. Enzymatic extract of *P. lilacinus* strains were evaluated semi-quantitatively for proteolytic activities on casein agar and gelatin agar, whereas to assess the chitinolytic activities, chitin agar was used. After 9 days of incubation, the proteolytic activity was highest in the extract of 14 days-old culture of *P. lilacinus* strain PIA#1 (Pandey *et al.*, 2011b).

Ganaie and Khan (2010) observed that efficacy of *P. lilacinus* against root-knot nematode infecting tomato significantly varied with the inoculation time. *P. lilacinus* applied simultaneously with *M. javanica* improved plant growth parameters, however, sequential inoculation of *P. lilacinus* 10 days prior to *M. javanica* was more effective than sequential inoculation of *M. javanica* 10 days prior to *P. lilacinus* in inhibiting the development of nematodes. In a microplot experiment, efficacy of *P. lilacinus* was evaluated against *M. incognita* at different inoculum levels and at different application times. At mid-season of the crop, early application of the fungus resulted in highest percentage of fungal infestation on the egg mass as compared to the plots that were treated with fungus 10 days before planting (Cabanillas and Barker, 1989).

Paecilomyces lilacinus can be cultured readily on various fungal growth media, however, these media are not suitable for the isolation of this fungus from soil. The reason of this drawback is the rapid growth of bacteria or other fungi on the growth media which either inhibits or obscures the presence of *P. lilacinus* (Mitchell *et al.*, 1987). Mitchell *et al.* (1987) amended a medium earlier developed by

Steiner and Watson (1965) for the isolation of various fungi from soil, plant and nematode tissue. The media was found suitable in the detection of *P. lilacinus* from soil or infected nematodes. Prabhu *et al.* (2008) conducted an experiment to evaluate spore production and formulation of *P. lilacinus* in different liquid media. Highest mycelia weight and spore load was found in semi-selective medium followed by 10% molasses. Fungus formulated with talc and fly ash proved the best carrier materials for mass production. Cabanillas *et al.* (1989b) prepared spores of *P. lilacinus* in five formulations, i.e. alginate pellets, diatomaceous earth granules, wheat grain, soil and soil + chitin. The viability of the fungus was high in wheat and granules, intermediate in pellets and low in soil and chitin-amended soil stored at 25±2°C. Egg mass parasitization was highest in the plots treated with pellets (32%) followed by chitin-amended soil (24%), wheat (16%), diatomaceous earth granules (12%) and soil (7%). Number of fungal colony-forming units per gram of soil was ten-fold greater than initial levels in the plots treated with alginate pellets.

Formulation and commercialization

Various formulations of *P. lilacinus* have been developed and used to control plant-parasitic nematodes. *P. lilacinus* strain 251 at 1×10^{10} spores/m^2 has been found effective in reducing the population of *Belonolaimus longicaudatus* on over-seeded bermudagrass (Crow, 2013). Commercial formulation of *P. lilacinus* (Phil. Strain No. 1) was developed as BIOCON by Asiatic Technologies Incorporation in Manila (Timm, 1987; Davide, 1990). In China, a formulation of *P. lilacinus* has been developed as 'Soybean Root Bio-Protectant' to control the soybean cyst nematode (Liu *et al.*, 1996). In South Africa, Pl Plus® is being sold as the commercial product of *P. lilacinus* for the management of root-knot and cyst nematodes (EPA, 2005; Viaene *et al.*, 2006). *P. lilacinus* strain 251 (PL 251) has been commercialized and registered for sale as BIOACT®WG for the control of nematode pests in several countries (Kiewnick, 2004; Atkins *et al.*, 2005). Moreover, PL 251 has received US EPA registration as a biological nematicide under the trade name Melocon®WG (EPA, 2005). NemaChek, Paecil, Paecio and PL Plus are the other products of *P. lilacinus* produced by different companies (Copping, 2004). Several companies and institutes in India have prepared a commercial product of *P. lilacinus* such as Yorker (Agriland Biotech Limited), Nematofree (International Panaacea Limited), *P. lilacinus* 1% WP (Indian Institute of Horticultural Research), PAECILO® (Agri Life), Gmax bioguard (Greenmax Agro Tech) and PAECILO™, which are available in the market and found effective against root-knot nematodes.

3.5.2 *Pochonia chlamydosporia* (= *Verticillium chlamydosporium*) (Goddard, 1913) Zare and Gams, 2001

Pochonia chlamydosporia is an opportunistic fungus that is parasitic on nematode eggs (Morgan-Jones *et al.*, 1983) and has a worldwide distribution (Domsch *et al.*, 1980). It is known to parasitize several phytoparasitic nematode species belonging to genera *Meloidogyne*, *Heterodera* and *Globodera* (Kerry, 1990), however strains of this fungal species vary in their efficacy to control nematode populations (Bourne *et al.*, 1994; Morton *et al.*, 2003; Mauchline *et al.*, 2004). The fungus was first reported as an egg parasite of nematodes in the UK by Wilcox and Tribe (1974). Later, its parasitic nature was demonstrated on cereal cyst nematode, *H. avenae* (Kerry and Crump, 1977; Kerry *et al.*, 1982, 1984). The root-knot and cyst nematodes are the primary hosts of this fungus, however, parasitism on citrus, burrowing and reniform nematodes have also been reported (Nagesh *et al.*, 2007). All stages of the fungus (hyphae, conidia, chlamydospores) occur in soil and actively growing mycelium infects eggs and females (Davies *et al.*, 1991). *P. chlamydosporia* can survive saprophytically in soil in the absence of its host. The fungus forms close contact with the eggshell, which leads to disintegration of eggshell and the vitelline layer and partial dissolution of chitin and the lipid layer (Lopez-Llorca and Duncan, 1988; Stirling, 1991; Saifullah and Thomas 1997). Egg hatching in the presence of fungus may be inhibited probably due to toxins secreted by the fungus (Morgan Jones *et al.*, 1983).

Taxonomy and morphology

TAXONOMIC POSITION
Division: Amastigomycota
Class: Deuteromycetes
Order: Moniliales
Family: Tuberculariaceae
Genus: *Verticillium*

MORPHOLOGY Based on its morphological and molecular characters, genus *Verticillium* has now been transferred to *Pochonia* (Zare and Gams, 2001; Zare *et al*., 2004). The colonies of *P. chlamydosporia* teleomorph *Metacordyceps chlamydosporia* are white in colour, which later becomes creamy but on the reverse side they appear creamy, pale yellow to orange. Phialides produce singly or in one or two whorls on aerial hyphae, slender and tapering towards the tip. *Pochonia* species abundantly produce dictyochlamydospores or some irregularly swollen hyphae (Moosavi and Zare, 2012), due to which colonies appear granular. These are produced in the aerial mycelium. As time advances, stalks become thick walled (Askary, 2008).

Mode of action

Pochonia chlamydosporia is a potential BCA of root-knot and cyst nematodes (Kerry, 1993; Atkins *et al*., 2004). The fungus parasitizes the eggs and adult females of plant-parasitic nematodes (Stirling, 1991; Kerry, 2000). Eggs in the early stages of embryogenesis are more susceptible as compared to the eggs containing J_2 (Manzanilla-Lopez *et al*., 2013). The fungus enters the nematode cysts either through natural openings or it may directly penetrate the wall of the cyst (Kerry, 1988a). Isolates and strains of *P. chlamydosporia* effectively colonize the rhizosphere and interact with the nematode development process (Cannayane and Jonathan, 2008). Observations of the infected nematode eggs showed that *P. chlamydosporia* forms a branched mycelia network in close contact with the smooth eggshell (Morgan-Jones *et al*., 1983; Lopez-Llorca and Duncan, 1988). The fungus produces an appressorium that adheres to the eggshell by mucigens and from which an infection peg develops that penetrates the eggshell (Segers *et al*., 1996). Penetration also occurs from lateral branches of the mycelium. Strains of *P. chlamydosporia* differ in host susceptibility. A difference in susceptibility has been observed when fungus parasitizes the eggs of root-knot and cyst nematodes. The fungal isolates obtained from the cyst nematode have the greater potential to parasitize the cyst nematode eggs as compared to the isolates recovered from root-knot nematodes (Viaene *et al*., 2006; Moosavi *et al*., 2010). This may be due to nematode host preference of the fungus (Manzanilla-Lopez *et al*., 2013). *P. chlamydosporia* infects female cyst nematodes that are exposed on the root surface for several weeks during their maturation. Depending on the length of time of fungal infection, effects on the fecundity and egg parasitism of the population of mature female cyst nematodes can be observed. But in the case of root-knot nematodes, where only the eggs are exposed in the rhizosphere and not the mature female, embryonic development and hatching completes within 10 days at 27–32°C. Hence, within a short span of time, root-knot nematodes are more prone to attack by *P. chlamydosporia* as compared to cyst nematodes.

It has been observed that the fungus causes disintegration of the eggshell's vitelline layer and also partial dissolution of the chitin and lipid layers, possibly due to the activity of exoenzymes. Serine proteases have been identified in *P. chlamydosporia* (Segers *et al*., 1994) and these extracellular enzymes are synthesized in the presence of nematode eggs and repressed by glucose. VCPI protease secreted by *P. chlamydosporia* has been found to hydrolyse the eggshell protein of *Meloidogyne* but not of *Globodera* (Segers *et al*., 1996) and this resistance to disintegration may be due to the thickness of eggshells of *Globodera*, which is as much as twice the thickness of *Meloidogyne* (Lopez-Llorca and Robertson, 1992). *P. chlamydosporia* also secretes a nematotoxin called phomalactone, which acts to enhance the pathogenicity (Segers *et al*., 1996; Viaene *et al*., 2006). Different strains of fungus might occupy separate niches in soil and rhizosphere due to differences in their enzymatic activities (Segers *et al*., 1996; Mauchline *et al*., 2004). There is much variation in the subtilisins produced by different isolates of *P. chlamydosporia* (Segers *et al*., 1999). In addition to the direct effects of

parasitism by *P. chlamydosporia* on the developing embryo, enzymatic effects on the eggshell may increase permeability and possibly facilitate the inward passage of toxins that may have been present in the environment. It has been suggested that *P. chlamydosporia* itself might produce such a toxin, because eggs did not hatch when the fungus was near eggs (Morgan-Jones *et al.*, 1983). However, Irving and Kerry (1986) obtained no evidence to support toxin production.

Under natural conditions, nematode eggs appear to be an important source of nutrients for *P. chlamydosporia.* The presence of root-knot nematodes in roots resulted in increased densities of *P. chlamydosporia* in the rhizosphere (Bourne *et al.*, 1996). *P. chlamydosporia* colonized the galled roots most extensively, however the fungus was least effective in controlling *M. incognita* at high nematode densities. The reason may be the retention of many egg masses embedded in the gall tissue, which protected them from fungal attack. Hence, control with this BCA is minimal unless the fungus is present on relatively poor hosts of the nematode on which only small galls are produced and most egg masses are exposed on the gall surface.

Ecology and distribution

Pochonia chlamydosporia is cosmopolitan in distribution. Arevalo *et al.* (2009) isolated *P. chlamydosporia* var. *chlamydosporia* and *P. chlamydosporia* var. *catenulata* from the eggs of root-knot nematode, *M. mayaguensis* and analysed them based on their cultural and morphological characteristics. The optimum temperature for growth and spore production for *P. chlamydosporia* isolates ranged between 24 and 28°C. Bourne and Kerry (2000) observed a difference in optimal temperature among the isolates of *P. chlamydosporia.* The fungus isolated from cyst nematodes was found to grow at lower optimal temperature as compared to that from root-knot nematode. Nagesh *et al.* (2007) evaluated four geographical isolates of *P. chlamydosporia* obtained from the soil samples of different agroclimatic conditions. It was observed that the strains differ in spore production, mycelial growth and pathogenicity at different pH and temperature.

Effect on phytonematodes

Biological control of root-knot nematodes, *M. incognita* and *M. javanica*, which are considered as a major limiting factor in crop production (Sharon *et al.*, 2007) can be done efficiently with the application of *P. chlamydosporia.* Tzortzakakis (2007) evaluated the effect of *P. chlamydosporia* at two different doses, i.e. 5000 and 60,000 chlamydospores/g soil against *M. incognita* at 450 and 1400 J_2/plant on tomato and pepper grown in pots. The results indicated a significant reduction in root-galling and juvenile density on pepper after 14 days. Application of *P. chlamydosporia* (5×10^3 chlamydospores/cm^3) in *Meloidogyne*-infested soil resulted in the colonization of egg masses by the fungus that varied from 16 to 43% and reduced the penetration of roots by J_2 (Nicole and George, 2000). Olivares and Lopez-Llorca (2002) investigated the presence of fungal egg parasites in Spanish soils infected with plant-endoparasitic nematodes. The most common was *P. chlamydosporia* var. *chlamydosporia* causing egg infection ranging from 70 to 100% and severity 35–40 hyphae/egg of *M. javanica. P. chlamydosporia* var. *catenulata* was proved as a potential BCA in an integrated pest management strategy for *M. incognita* in vegetable crops in Cuba (Garcia *et al.*, 2004). The presence of *P. chlamydosporia* was associated with a reduction in the number of plant-parasitic nematodes (51–78%) including the migratory ectoparasites, whereas free-living nematodes, culturable bacteria and bacterial populations were unaffected by the application of fungus (Tahseen *et al.*, 2005). De Leij *et al.* (1992a) also reported that treatments with *P. chlamydosporia* decreased the number of eggs, juveniles and galls in tomato grown in pots inoculated with *M. incognita*. Soil population of *M. hapla* in tomato root zone decreased by more than 90% in sandy loam soil applied with *P. chlamydosporia* alone or with aldicarb (de Leij *et al.*, 1993). Wang *et al.* (2005) demonstrated *P. chlamydosporia* parasitizing the eggs and suppressing the population of the reniform nematode, *R. reniformis* under *in vitro* and greenhouse conditions, respectively. Application of *P. chlamydosporia* colonized the egg masses of *M. hapla* from 16 to 43% in lettuce and reduced the nematode population at

densities below 8 eggs/cm^3 soil (Viaene and Abawi, 2000). A 96% egg parasitism of *M. incognita* by *P. chlamydosporia* has been observed in laboratory conditions (Dhawan and Singh, 2010). Van Damme *et al.* (2005) tested the efficacy of *P. chlamydosporia* against *M. incognita* in two cropping systems, i.e. lettuce and tomato, under field conditions. It was concluded that one-time application of *P. chlamydosporia* was able to slow down the population build-up of root-knot nematode for at least 5–7 months. Application of *P. chlamydosporia* at 2 kg/ha in pigeonpea microplots against *Heterodera cajani* for 3 years resulted in a significant increase in crop yield and reduction in nematode population (Kumar and Prabhu, 2008). Yang *et al.* (2012) isolated three strains of *P. chlamydosporia* var. *chlamydosporia* from *M. incognita*-suppressive soil and then genetically characterized them with multiple *Pochonia*-selective typing methods based on analysis of β-tubulin, rRNA internal transcribed spacer (ITS), rRNA small subunit (SSU) and endobacterial repetitive intergenic consensus (ERIC) PCR. All the three strains used in the experiment significantly reduced the numbers of egg masses of root-knot nematode.

Formulation and commercialization

Considerable efforts have been made to prepare a commercial formulation of *P. chlamydosporia* and as a result various strains of this fungus have been successfully commercialized in Europe, America and Africa. In Italy, IPP-21, an isolate of *P. chlamydosporia* containing mycelium, conidia and chlamydosporia based on oil emulsion, has been commercialized for some years under a registration-free permission (Manzanilla-Lopez *et al.*, 2013). Xianchongbike, a formulation of *P. chlamydosporia*, has been commercialized in China (Mo *et al.*, 2005). In Cuba, KlamiC®, a chlamydospore-based product of *P. chlamydosporia* var. *catenulata*, is being used successfully as a soil application in peri-urban agriculture (Montes de Oca *et al.*, 2009; Fernández-Larrea, 2012). Kerry (1988b) was successful in growing approximately 1 cm hyphae of *P. chlamydosporia* on alginate granules, which suggests that such granular formulations may be suitable for application against nematodes. Coosemans (1988) reported establishment of *P. chlamydosporia* in field soil after it was grown on a substrate and incorporated into a peat growth medium. Galling caused by root-knot nematode was substantially reduced when *P. chlamydosporia* grown on oat kernels was introduced into field soil at 0.5% and 1% w/w (Godoy *et al.*, 1983; Rodriquez-Kabana *et al.*, 1984). *P. chlamydosporia*-enriched vermicompost at 50 g/m^2 has been found effective in sustainable management of *M. incognita.* The formulation proved effective in reducing the root-knot index, soil and root population of nematodes and increasing the colonization of *P. chlamydosporia* on the roots (Chaya and Rao, 2012).

3.5.3 *Trichoderma harzianum* Rifai 1969 (Teleomorph: *Hypocrea albofulva* Berk. and Br. 1873)

Trichoderma harzianum is a potential egg parasite of root-knot nematodes and other plant-parasitic nematodes (Dos Santos *et al.*, 1992; Hafeez *et al.*, 2000; Askary, 2008; Khattak *et al.*, 2008). Several poisoning and antibiotic compounds are produced by *T. harzianum* (Di Pietro, 1995), which protect the plant from plant-parasitic nematodes present in the soil (Meyer *et al.*, 2000; Haggag and Amin, 2001; Santosh *et al.*, 2005). The fungus colonizes near the plant roots and grows on the roots, thereby making a physical barrier for the invading nematodes (Inbar *et al.*, 1994). In addition, *Trichoderma* help the plant in tolerance to stress conditions by enhancing root development. It also participates in solubilization of inorganic nutrients (Wickramaarachchi and Ranaweera, 2008), thus lessening the requirement for man-made nitrogen fertilizers (Harman, 2000).

Taxonomy and morphology

TAXONOMIC POSITION
Division: Ascomycota
Class: Sordariomycetes
Order: Hypocreales
Family: Hypocreaceae
Genus: *Trichoderma*

MORPHOLOGY The genus *Trichoderma* is characterized by fast-growing hyaline colonies bearing repeatedly branched hyaline conidiophores

which appear in tufts and are smooth-walled, straight or flexuous. Phialides are divergent and flask-shaped. Conidia are hyaline or more usually green, smooth or roughened, with shapes ranging from subglobose to ovoid or short ellipsoid. Chlamydospores are fairly abundant, intercalary and are terminal on short branches (Askary, 2008).

Mode of action

Direct parasitism of nematode eggs due to increase in extracellular chitinase activity and induced plant defence mechanisms leading to systemic resistance are the two main suppression mechanisms attributed to *T. harzianum* (Sahebani and Hadavi, 2008). The hyphae of *T. harzianum* parasitize eggs and larvae of *M. incognita* by dissolving the chitin layer through enzymatic activity and penetration of the eggs and larval cuticle. The hyphae proliferate within the organism and produce toxic metabolites (Dos Santos *et al.*, 1992). The enzymes produced by *Trichoderma* spp. such as chitinase, glucanases and proteases play an active role in parasitizing the host (Haran *et al.*, 1996). The enzyme chitinase has chitinolytic activity (Lorito *et al.*, 1993) and since the outer shell of the nematode's egg is made of chitin, the eggs are greatly affected by the application of *Trichoderma* spp. (Haggag and Amin, 2001; Jin *et al.*, 2005). *T. harzianum* produces extracellular chitinase and proteinase because of the proteinaceous and chitinase nature of nematode eggshell. Other extracellular protein is induced by colloidal chitin, which may be involved in nematode egg penetration. Antibiotics such as trichodermim, dermadin, trichoviridin and sesquiterpene heptalic acid are produced by *Trichodermum viride*, which are involved in the suppression of nematodes (Jonathan, 2010). Prasad and Anes (2008) reported that ethyl acetate and methanol extracts of *T. viride* and *T. harzianum* significantly reduced the root galling caused by *M. incognita* in okra. In a laboratory study it was found that antifungal metabolites of *Trichoderma* spp. prevented the hatching of *M. incognita* eggs and the growth of J_2. Light microscope observations showed the evidence that mycoparasitism contributed to the aggressive nature of the tested isolate of *T. viride* NRRL 6418 and *T. harzianum* (*Hypocrea lixii* TWCI) against *M. incognita* female body and egg masses (Jegathambigai *et al.*, 2011).

Ecology and distribution

Trichoderma harzianum is ubiquitous, having a worldwide distribution. The fungus can be found in common soil, litter and on various materials including paper and tapestry. The optimum temperature for colonization of the fungus has been found to be 15–21°C, however, under *in vitro* conditions, the temperature ranges between 27 and 30°C. The fungus activity is severely limited at 3 or 39°C (Eastburn, 1986).

Effect on phytonematodes

Several isolates of *T. harzianum* have been reported to reduce the infestation of root-knot nematodes (Spiegel and Chet, 1998; Khattak and Khattak, 2011). Eapen and Venugopal (1995) demonstrated that isolates of *Trichoderma* spp. have a broad spectrum of biocontrol activity against a number of pathogenic fungi and nematodes. A serine protease of 28 kDa with trypsin activity was isolated from *Trichoderma* strain 2413. The enzyme caused reduction in the number of hatched eggs of root-knot nematodes and exhibited synergistic effects with other proteins produced during antagonistic activity of the strain. There was a significant reduction in the number of hatched eggs of the root-knot nematode, *M. incognita* when incubated with pure PRAI (trypsin-like protease) preparations of *T. harzianum* CECT 2413 (Suarez *et al.*, 2004). Efficacy of *T. harzianum* tested under *in vitro* conditions resulted in parasitization of 53% eggs of *M. incognita* (Dos Santos *et al.*, 1992). Pant and Pandey (2001) reported maximum reduction in the population of *M. incognita* by *T. harzianum* applied in sterilized soil at 5000 spores/pot. Windham *et al.* (1989) tested the effect of *T. harzianum* applied before maize seeding in small pots filled with *M. arenaria*-infested soil. After 50 days, top and root fresh weights had increased, with a decrease in the number of eggs per gram root, as compared to the control plants. Kumar and Prabhu (2008) applied *T. harzianum* at 5 kg/ha

against pigeonpea cyst nematode, *H. cajani* and found a significant reduction in nematode population and increase in the yield of pigeonpea. A greenhouse study was undertaken for evaluating *T. viride* and *T. harzianum* as seed treatment of okra against root-knot nematode, *M. incognita*. The observations recorded 45 days after sowing indicated an increase in plant growth parameters and reduction in the population of nematodes (Kumar and Jain, 2010). Muthulakshmi *et al.* (2010) evaluated the efficacy of *T. viride* against *M. incognita* in mulberry. The fungus was able to control nematode population and improve the mulberry leaf yield. Direct interactions between *T. harzianum* and potato cyst nematode *G. rostochiensis* under *in vitro* conditions resulted in death of the juveniles of those cysts and eggs which were penetrated by the fungus (Saifullah and Thomas, 1996). In greenhouse experiments *Trichoderma* was evaluated against *M. javanica* infecting tomato plants. The results indicated reduction in root galling and increase in top fresh weight of tomatoes infested with nematode following soil pre-treatment with *Trichoderma* peat–bran preparations. The use of a proteinase Prb1-transformed line (P-2) containing multiple copies of this gene improved biocontrol activity in the greenhouse experiments as compared to non-transformed wild-type strain (Sharon *et al.*, 2001). All the strains of *Trichoderma* used in the experiment colonized the eggs and J_2 of *M. javanica.* Naserinasab *et al.* (2011) observed increased activities of soluble peroxidase (SPOX) in *T. harzianum* BI + salicylic acid-treated tomato inoculated with *M. javanica*, which led to the conclusion that besides direct antagonism, induction of defence- related enzymes involved in the peroxidase pathway contribute to enhance resistance against the attack of *M. javanica* on tomato. Saifullah (1996a,b) reported 100% death of *G. pallida* and *G. rostochiensis* after 24 h of exposure with the poisoning compound of *T. harzianum* on the medium. Soil drenching of *Trichoderma* has also been found useful in reducing the population of nematodes. Drenching of conidia suspension of *T. viride* NRRL 6418 and *T. harzianum* (*Hypocrea lixii*) at 1×10^{14} spore/ml significantly reduced the root-knot nematode disease in *Livistona rotundifolia* and increased the plant growth (Jegathambigai *et al.*, 2011).

Formulation and commercialization

In recent years several companies of the world have prepared commercial formulations of *Trichoderma* spp. such as Mycolab (commercial product of *T. lignorum* prepared by Laboratórios Laverlam, Columbia), Trichobiol (commercial product of *T. harzianum* prepared by Control Biológico Integrado; Mora Jaramillo Arturo Orlando – Biocontrol, Columbia), Trifesol (commercial product of *T. viride* strain 2684 prepared by BioCultivos S.A., Bogotá, Colombia) and Commander Fungicide (commercial product of *T. harzianum* prepared by HTC Impex Private Limited, India) (Woo *et al*., 2014). ECOSOM®, a biological nematicide based on a selected strain (IIHR-Th-2) of *T. harzianum*, has been prepared by Agri Life, India and is being used for the management of root-knot and reniform nematodes. *T. harzianum* strain MTCC-3841 (NBRI-1055) spores scraped from potato dextrose agar plates was formulated as 11–12 $\log^{10}$ CFU/g by Singh and Nautiyal (2012), which provided an extra advantage of smaller packaging for storage and transportation, thereby cutting the production cost. Seed application of the formulation resulted in increased plant growth.

3.5.4 *Aspergillus niger* (van Tieghem, 1867)

Aspergillus niger is an asexual saprophytic fungus growing on dead and decaying plant material, stored grain and compost piles. The fungus has a nematotoxic activity. The nematicidal substance produced by the fungus has an inhibiting effect on the nematodes. The fungus also has the ability to colonize the nematode eggs and cysts, thus providing early protection to the growing plants against nematodes.

Taxonomy and morphology

TAXONOMIC POSITION
Division: Ascomycota
Class: Eurotiomycetes
Order: Eurotiales
Family: Trichocomaceae
Genus: *Aspergillus*

MORPHOLOGY Hyphae, septate and hyaline conidial heads radiate initially, splitting into

columns at maturity. Conidiophores are long, smooth and hyaline, darker at the apex and terminate in a globose vesicle. Phialides undergo blastic basipetal conidiogenesis to produce black globose mitospores. *A. niger* can be distinguished from the other species of the genus on the basis of spores that are carbon black or very dark brown produced from biseriate phialides (Raper and Fennell, 1965).

Mode of action

Aspergillus niger is an egg parasite of nematodes. As soon as the fungus comes in contact with a cyst or an egg mass, it rapidly grows and colonizes those eggs where larval formation has not been completed. However, when the larva is formed, the egg becomes less vulnerable. It has been suggested that this differential vulnerability of the egg is due to chitinolytic activity of the fungus. Chitin is a major constituent of the eggshell, which the larval cuticle lacks. In some cases there is enzymatic disruption of nematode structural elements such as eggshell and larval cuticle or physiological disturbance due to biosynthesis of diffusible toxic metabolites (Jatala, 1985, 1986).

Ecology and distribution

Aspergillus niger is a ubiquitous group of filamentous fungi, having widespread distribution and can be found in a broad range of habitats, i.e. soil, plant debris and indoor air environment. Conidiospores are distributed by air (Schuster *et al*., 2002) and the spores are deposited when they come into contact with a solid or liquid surface and start germination on finding favourable moisture. The fungus can sustain growth even in freezing temperatures, however optimum temperature range for its growth is 35–37°C. Growth of the fungus can take place at a wide range of pH, i.e. 1.4–9.8. The normal indoor conditions for the growth of *A. niger* has been found at a temperature of 30°C and relative humidity for maximum growth at 75% (Ababutain, 2013).

Effect on phytonematodes

Toxicity of culture filtrate of *A. niger* on eggs and juveniles of *Meloidogyne* spp. has been observed with 93.3% juvenile mortality when treated with 20-times diluted culture filtrate of the fungus (Dahiya and Singh, 1985). Singh *et al.* (1991) reported lesser damage to tomato by *M. javanica* in the presence of *A. niger*. Zareen *et al.* (2001b) tested the efficacy of filtrates of seven species of *Aspergillus* against *M. javanica* infesting tomato. They found all the isolates suppressive to nematodes but a few isolates, i.e. AnC_2, AnR_3 and AnM_1, were highly antagonistic to nematodes. In another experiment, secondary metabolites of *A. niger* have been found to reduce the nematode populations and root-knot index and promoting the plant growth of tomato (Li *et al*., 2011).

Formulation and commercialization

Some commercial formulations of *A. niger* have been prepared by different companies both for soil application as well as seed treatment. In India, Kalisena SL, Pusa Mrida and Kalasipahi (capsules) are meant for soil application, whereas Kalisena SD and Beej Bandhu, for seed dressing. Except for Beej Bandhu, all the formulations have an extraordinarily long shelf life of more than 2 years at 15–35°C when packed in polythene bags and stored under less than 80% RH. Application of Kalisena SD has been found to increase the germination and seedling vigour of a number of crops (Sen, 2000). The formulations are effective in acidic and alkaline soils, under high and low moisture conditions at a soil temperature ranging between 18 and 45°C.

3.6 Toxin-producing Fungi

Some fungal species exhibit a range of antagonistic activities against nematodes which include production of nematotoxic compounds (Siddiqui and Mahmood, 1996; Kerry, 2000; Llopez-Llorca and Jansson, 2006; Table 3.4). These fungi directly parasitize nematodes or secrete nematicidal metabolites causing an adverse effect on the viability of one or more stages (Llopez-Llorca and Jansson, 2006). Fungi belonging to the Pleurotaceae family of mushrooms utilize adhesive knobs to catch nematodes. Once the nematode is caught, the fungus secretes a nematicide that kills the

Table 3.4. Species of some toxin producing fungi and their toxic compounds.

Fungi species	Taxonomic classification	Toxins
Conocybe lactea	Basidiomycetes	Phalloidin
Coprinus comatus	Basidiomycetes	O-containing heterocyclic compounds
Pleurotus ostreatus	Basidiomycetes	*trans*-2-decanedioic acid
P. plumonarius	Basidiomycetes	linoleic acid, *p*-anisoylalcohol, *p*-anisaldehyde, *S*-coriolic acid, 1-(4-methoxyphenyl)-1,2-propanediol, and *Z*-hydroxy-(4′-methoxy)-propiophenone

nematode and prevents its escape. Toxic metabolites produced by fungi may either kill the nematodes or disrupt their parasitic activities by causing a change in the physiology of the nematode. *Pleurotus ostreatus* produces droplets of a potent toxin that quickly immobilizes nematodes (Thorn and Barron, 1984). There are some metabolites which act as hatching stimulants that may affect eggshell permeability and lead to premature hatching.

There are several such reports of fungi producing nematicidal compounds (Li *et al.*, 2007; Anke, 2010), however no major breakthrough has been achieved so far regarding commercial production of these fungal compounds for widespread use (Li *et al.*, 2007).

3.7 Integrated Management

There are different management methods that may be adopted, depending on the feasibility, to control plant-parasitic nematodes and to improve the crop yield. However, it has been frequently observed that a single method has certain limitations in nematode management and is not always found to provide adequate and economic control of nematodes. Therefore, integration of various suitable management options may prove helpful in developing an environmentally sustainable, economically viable and practically feasible approach of nematode management. Under this situation, an integrated approach consisting of BCA in conjugation with a lower dose of pesticide and/or any plant products may provide effective control, if the components are mutually compatible.

Simon and Pandey (2010) applied *P. lilacinus* in combination with *P. chlamydosporia*, which resulted in a significant reduction in root galling on okra plants. *P. chlamydosporia* in association with *T. harzianum* has been reported to significantly reduce the cyst nematode population in pigeonpea (Kumar and Prabhu, 2008). Combined application of *P. chlamydosporia*, *T. harzianum* and *Glomus mosseae* proved more effective in controlling the population of cyst nematode, *H. cajani* on pigeonpea than either of the treatments alone (Siddiqui and Mahmood, 1996). Oduor-Owino (2003) applied *P. lilacinus* with aldicarb and chicken manure against root- knot nematodes. The combination significantly suppressed the gall development on plant roots and the nematode population in soil.

Application of fungal antagonist in combination with parasitic bacteria has been found effective in controlling the population of plant-parasitic nematodes. Synergistic application of *P. chlamydosporia* and parasitic bacteria, *Pasteuria penetrans* resulted in reducing the root-knot nematode population on tomato with greater efficacy than either of the BCA used alone (de Leij *et al.*, 1992b). Combined application of *T. viride* and antagonistic bacteria, *Pseudomonas fluorescens* in soil each at 10 g/plant significantly reduced the root-knot nematode disease and improved the growth of mulberry (Muthulakshmi *et al.*, 2010).

Pochonia chlamydosporia in combination with a nematicide, carbofuran and a botanical, neem-seed cake suppressed the root-knot disease severity in okra. Root galling, egg mass production and juvenile population was significantly reduced, ultimately leading to an increase in yield of the crop (Dhawan and Singh, 2009). De Leij *et al.* (1993) reported that *P. chlamydosporia* when applied in combination with aldicarb at 2.8 kg/ha did not affect the activity of the fungus and provided better control of *M. hapla* than when treated with aldicarb or *P. chlamydosporia* alone. Muthulakshmi *et al.* (2012)

conducted a pot experiment under greenhouse conditions to study the effect of *P. chlamydosporia* individually and in combination with carbofuran in the management of *Globodera* sp. in potato. A significant decrease in the number of cysts, eggs and females were recorded in both combined as well as individual treatment as compared to control. However, combined treatment proved superior over individual treatment in reducing the number of cysts and eggs as compared to the untreated control. Recently, Chandel *et al.* (2014) applied soil fumigant metham sodium in combination with neem cake enriched with *P. lilacinus* against root-knot nematode *M. incognita* infecting tomato, capsicum and carnation under polyhouse conditions. The results indicated reduction in root galling and increase in crop yield. Hemlata *et al.* (2002) studied the effect of *T. harzianum* and neem cake alone and in combination to manage *M. incognita* in chickpea cv. Type-3. Greatest reduction in the population of *M. incognita* was recorded with the combined application of neem cake and *T. harzianum*, followed by neem cake and *T. harzianum* alone. *T. harzianum* in combination with neem cake resulted in reducing the population of citrus nematode, *Tylenchulus semipenetrans* (Parvatha Reddy *et al*., 1996). Cannayane and Rajendran (2001) applied *P. chlamydosporia* at 20 g/plot (6×10^7 CFU/g substrates) along with *P. lilacinus* and neem cake. The results indicated an effective control of *M. incognita* along with 58% increase in yield of brinjal plants. Application of fungal antagonists with organic manure has also been found effective in reducing the population of plant-parasitic nematodes. Farmyard manure enriched with *A. niger* and *P. lilacinus* along with neem cake have been found effective in suppressing the population of root-knot nematode and increasing the yield of chickpea (Singh *et al.*, 2011).

NC FLO, a commercial product of Biogrow Chemicals Private Limited, is being sold in the market and has been found effective against plant-parasitic nematodes. This product is a mixture of four fungal strains, *A. oligospora*, a nematode-trapping fungus, *H. rhossiliensis*, an endoparasite of nematode, *Acremonium butyri*, a fungus causing nematode ovicide and *P. lilacinus*, the nematode egg- and female-parasitic fungus. Application of integrated module may be suitably manipulated, exploited and commercialized for the management of plant- parasitic nematodes. However, implementation of INM module requires adequate demonstration in farmers' field and educational input to the growers during extension services.

3.8 Conclusions and Future Prospects

Considering environment safety, human health hazards and cost of management, fungal BCA is the best option, much safer and highly practicable. However, biological control of plant-parasitic nematodes through nematophagous fungi sometimes provides erratic results particularly under field conditions because the soil ecosystem is very complex. Kerry (1979) is of the opinion that even if these parasitic fungi are cultured artificially and added to soil where they are absent, it may take a few years for them to increase the population up to the level that is needed for the control of the nematode population. Therefore, during this period the growers would have to use other control measures such as tolerant varieties or nematicides. However, once established in soil, such parasitic fungi will prove as effective as a resistant cultivar or an efficient nematicide in limiting the nematode population.

In the past few decades extensive surveys of nematophagous fungi have been carried out all over the world in several countries, but a large number of these fungi are yet to be discovered. Furthermore, research on the identification of new discoveries and their exploitation against economically important plant-parasitic nematodes are greatly needed. In the recent years, scientists have had success in commercially exploiting a few BCAs such as *P. lilacinus*, *P. chlamydosporia*, *T. harzianum*, *A. niger* and *A. oligospora* against plant-parasitic nematodes but no fungal BCA proved promising from all the angles. If a fungal BCA is successful in controlling one group of nematodes, the problem of the other group remains unsolved. *P. lilacinus* has been found in practice as a good colonizer (Cabanillas *et al.*, 1988) as they attack young females and the egg masses which are embedded in roots. But on the other hand they lack a mechanism

of aggressive trapping or attachment device, hence their success in controlling mobile nematodes is very limited (Esser and El-Gholl, 1993). It has been commonly observed that if two or more phytoparasitic species of nematodes are feeding on one plant host, *P. lilacinus* can limit or check the population of only one species, therefore the problem of other mobile nematodes will remain unchanged. In addition, isolates of fungal BCAs differ widely in virulence and ability when established in soil and therefore their results under field conditions are too erratic. Another drawback is the presence of antagonists of these fungi in soil, which often results in the failure of fungal BCAs when applied in the field.

It is true that use of fungal BCAs is environmentally safe and a right approach in the management of plant parasitic nematodes but it is difficult to say that they are a substitute for nematicides. Nematophagous fungi may not control the nematodes when the inoculum level of the latter is too high in soil, however the population of the nematode can be reduced to an extent that would ultimately result in reducing the crop yield loss. Therefore, the approach should be to apply these fungal BCAs in conjugation with a resistant cultivar, plant product or include them in crop rotation to make a part of an INM programme. In my opinion, combined seed treatment with fungal BCA, botanical and pesticide seems one of the best options as it is economical, much safer and can prove highly practicable under field conditions. But before going for such option, a compatibility test of the fungal BCA with the pesticides and botanicals should be ascertained to ensure that none of the components involved in the combined treatment are mutually suppressed. Application of this integrated module may be suitably manipulated, exploited and commercialized for the management of plant-parasitic nematodes; however, before commercialization of any such INM module, multi-locational field trials in farmers' field should be conducted to demonstrate the results and educate the growers during extension services.

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Trade names or commercial products mentioned in this chapter are solely for the purpose of providing specific information and does not imply recommendation or endorsement.

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4 Nematophagous Fungi: Ecology, Diversity and Geographical Distribution

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4.1 Introduction

An organism lives in a state of dynamic equilibrium with the environment. Interactions of an organism with members of its community are intraspecific and interspecific. The ecosystem comprises the biotic community and the non-living environment and is the basic functional unit, as it includes both the organism and the environment, each influencing the properties of the other and both necessary for the survival and maintenance of life. Climate is the primary environmental factor influencing the nature of flora and fauna in a region. The climatic components are temperature, rainfall and relative humidity. The physical, chemical and biological conditions of soil are secondary to the climate. But they all determine the population, seasonality and determine the survival and population fluctuations of soil micro- and meso-biota in particular. Climate change is slow, steady and erratic. It has impact on local weather and other phenomena, as well as on ecosystems, climatic, ecological and geographical regions.

Natural ecosystems are aquatic and terrestrial. Aquatic ecosystems include ponds, lakes, rivers and oceans; terrestrial ecosystems include grasslands, forests, deserts and tundra. The components of the non-living environment in their elements and compounds are air, water, soil and organisms. Any environmental factor potentially unfavourable to organisms can act as *stress*. Resistance to stress is defined as the ability of living organisms to survive in a given environmental complex that depends upon its evolutionary history leading to its adaptation. A body remains in a state of *strain*. Biogeography refers to distribution of microbes, plants and animals. Specific organisms are restricted to specific communities or groups of communities. Three aspects of distribution of an organism are generally recognized: (i) the geographical range – the specific extent of the area where the organism naturally occurs; (ii) the geologic range – the distribution in respect of time, past or present; and (iii) the ecological distribution – major biotic communities (e.g. biomes of marine, freshwater and terrestrial ecosystems).

Usually, animal and plant communities are studied separately, which obscures the wholeness of a community and limits human understanding of the same. This approach of ecology is called synecology (study of groups of organisms in relation to their environment; includes populations, community, and ecosystem ecology with energy flow through the

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ecosystem), while the approach for the considerations of any single organism is called autoecology (ecological study of the individual organism in relation to its environment). A more inclusive classification is the one such as between animals and plants. This classification is called a *biome*. The biome is a broad ecological unit characterized by uniformity and distinctive life forms of the climax species, to whichever group(s) it may belong. Here the emphasis is on the life forms and their communities. A community is a continuum through time and space. Any large area is so complex that it must be separated into subdivisions. The aggregation of organisms in a given locality or habitat must be regarded as a unit while studying a community. Classification systems, therefore, are based on physiognomy, habitat, floristic characteristics like species composition and dominance and community dynamics. Physiognomy implies general appearance. It helps in naming and delimiting communities, such as during surveys and as the basis in further subdivision. Habitats may be well-defined and physiography is used to classify and name communities such as sand dunes, cliffs, tidal mudflats, lakes, ponds and streams.

Nematode-destroying fungi are now more commonly and aptly called nematophagous (from Greek *nemat-* or *nemato* = thread + *phag* – from Greek *phagos* – *phagous* = to eat, to ingest) fungi. Nematophagous fungi (NF) are increasingly being used for control of plant and animal parasitic nematodes. Swe *et al*. (2011) have reviewed nematode-trapping fungi (NTF), which, according to them include: (i) NTF using adhesive or mechanical traps; (ii) endoparasitic fungi; (iii) egg-parasitic fungi; and (iv) toxin-producing fungi (Kendrick, 2001; Liu *et al*., 2009). Nordbring-Hertz and Tunlid (2000) grouped NF into three categories according to their mode of infestation: NTF, endoparasitic and toxic-compound-producing (NPF). It is going a little too far to include egg-parasitic and toxin-producing nematophagous fungi (NPF) into NF. Further, there is no mention of 'snare' in connection with NTF. Duddington (1956) refers to a 'snare' as the type that gives out an adhesive knob as in *Dactylella ellipsosora* (Discomycetes, Ascomycetes), a hyperparasite. The knob penetrates with a penetration peg into the nematode parasite and spreads its hyphal network into the whole of the nematode, which is killed in the process (Dasgupta, 1988). NPF has been gaining importance over several decades for fundamental studies on their distribution, ecology, systematics and biocontrol potential of nematode pathogens of plants and animals (Li *et al*., 2005). Cobb (1917) suggested that NF might serve as biocontrol agent for management of plant-parasitic nematode. Linford (1937; Linford *et al*., 1938) possibly used NF for biological control of nematodes for the first time. NF are broadly grouped based on their mode of action into nematode-trapping (predaceous), endozoic (endoparasitic), toxin-producing and egg-and female-parasitic (opportunistic). Predaceous fungi are basically nematode trappers, belonging to the Zygomycetes and Deuteromycetes group. More than 100 species of fungi are reported to be predaceous. Fungi such as *Monacrosporium cionopagum* form sticky branches, *Arthrobotrys oligospora* forms a sticky network. *Dactylella ellipsospora* forms sticky knobs, *Dactylaria candida* forms non-constricting rings, *Dactylaria brachopaga*, *Dactylella bembicodes* and *Arthrobotrys dactyloides* form constricting rings and *Stylopage grandis* forms a sticky mycelium and catches the nematode moving beside them. The NTF, *Arthrobotrys* spp. and *Monacrosporium* spp., trap nematodes in constricting rings and adhesive nets, respectively (Duponnois *et al*., 1998, 2001; Stirling and Smith, 1998; Stirling *et al*., 1998; Viaene and Abawi, 1998; Kumar and Singh, 2006b; Thakur and Devi, 2007). These fungi naturally occur in soils at low concentrations, and predate only some specific nematodes, which limits their potential use. Their recognition mechanism involves interaction between a lectin from fungus and a carbohydrate moiety of nematode cuticle (Nordbring-Hertz and Mattiasson, 1979). In France, formulations of *Arthrobotrys irregularis* isolates were commercialized as Royal 300 for the control of *Ditylenchus myceliophagus* and root-knot nematodes (Cayrol and Frankowski, 1980; Cayrol, 1983, 1988). Soil-inhabiting nematodes are frequently infected by the various fungi that naturally suppress the soil nematode population. Stirling (1988) reviewed the biocontrol of plant-parasitic nematodes

and indicated the possibility of exploitation of soil antagonists for the management of nematodes. Root-knot nematode species including *Meloidogyne incognita* (Duponnois *et al*., 1996; Kumar and Singh, 2006a; Thakur and Devi, 2007), *Meloidogyne javanica* (Khan *et al*., 2006) and *Meloidogyne hapla* (Viaene and Abawi, 1998) are frequently attacked by fungi in vegetable crop ecosystems. NF were found associated with rice root-knot nematode, *Meloidogyne graminicola* and *A. dactyloides* and *Dactylaria brochopaga* are effective to control the nematode infecting rice (Singh *et al*., 2000). Yang *et al*. (2007a) reported a novel serine protease from *Dactylella varietus* found to have a potential role in infection against nematodes.

Over 50 species of endozoic fungi belonging to diverse groups such as Blastoeladiales, Chytridiales, Entomophthorales, Zoopagales, Moniliales, imperfect Ascomycetes and imperfect Basidiomycetes have been recovered from nematodes. Most of the fungi require a nematode host to complete their life cycle, thereby controlling nematodes. Among the endozoic fungi, Moniliales, *Acrostatagmus*, *Harposporium* and *Meria* are frequently encountered in crop fields and have been cultured axenically. Some endozoic fungi such as *Meria contospora*, *Hirsutella rhossiliensis* and few members of *Verticillium* and *Cephalosporium* produce adhesive spores, which become firmly attached to nematode cuticle, produce a germ tube and finally penetrate into the nematode body and destroy the body contents. *M. contospora* and *Catenaria anguillulae* have been experimentally used to reduce the population of cyst and other nematodes. Thus, endozoic fungi are more promising over predaceous fungi as they are culturable, some bear adhesive spores, have a long dormancy period, depend on the nematode to complete their life cycle and are equipped with specialized infective structures.

The egg- and female-parasitic (also grouped as opportunistic) fungi are promising groups for control of various nematodes. Most promising ones are *Paecilomyces lilacinus*, *Pochonia* (=*Verticillium*) *chlamydosporia*, *Dactylella oviparasitica*, *Nematophthora gynophila* and *Cladosporium oxysporum* (Khan and Goswami, 2001). *P. lilacinus* has a wide geographical distribution and was initially isolated from the egg masses of *M. incognita acrita* found infecting potato roots in the central Peruvian highlands in Huanuco valley (Jatala *et al*., 1979; Jatala, 1982, 1986). It was found effective in colonizing the eggs of *Globodera pallida* and *M. incognita* and significantly reduced the nematode population in soil (Jatala *et al*., 1979). Similarly, *P. chlamydosporia* was proved as a potential parasite of eggs of *Meloidogyne arenaria* and caused a reduction of the nematode population (Kerry, 1984; Rao, 2007). Among the facultative egg-parasitic fungi, *P. lilacinus* is one of the most effective and successful biocontrol agents, and has been commercialized as Biocon in the Philippines and Yorker/Bionematon in India for several nematodes including root-knot nematodes in vegetables (Khan and Goswami, 2002; Verdejo-Lucas *et al*., 2003; Goswami and Mittal, 2004; van Damme *et al*., 2005; Goswami *et al*., 2006; Haseeb and Kumar, 2006; Kumar *et al*., 2009) and plantation crops like coffee and banana. This nematode is well adapted to tropical and subtropical climates but less well adapted to temperate soil conditions. *P. chlamydosporia* is an effective egg-parasitic fungus of root-knot nematodes, but it prefers mild temperate soil conditions (Atkins *et al*., 2003). Recently, Pendse *et al*. (2013) reviewed NF, giving its present status and future prospects for controlling plant-parasitic nematodes. While discussing the early history of NF, three periods were elaborated: (i) period of taxonomy and ecology; (ii) period of development and understanding trapping mechanism and physiology of nutrition; and (iii) period to establish biocontrol against parasitic nematodes (see details in Pendse *et al*., 2013).

4.2 Diversity and Taxonomy

Fungi represent the fifth kingdom in the living organisms (Kendrick, 2001). NPF are found in all major groups of fungi, such as lower Oomycetes, Chytridiomycetes, Zygomycetes and higher fungi such as Ascomycetes, Basidiomycetes and Deuteromycetes.

4.2.1 Ecological speciation

Speciation results from evolution of one species into two and is one of the most fundamental problems to appreciate in biology. Modes of speciation in fungi have been reviewed (Natvig and May, 1996; Burnett, 2003; Kohn, 2005; Giraud *et al*., 2008). Rundle and Nosil (2005) have defined 'ecological speciation' as the 'process by which barriers to gene flow evolve between populations as a result of ecologically based divergent selection'. Fungi are considered excellent models for investigating eukaryotic speciation, in general (Burnett, 2003; Kohn, 2005), although not included in general reviews. According to Giraud *et al*. (2008), the reasons for the fungi being excellent models for eukaryotic speciation are: (i) many fungi can be cultured *in vitro* and their mating types and their genetics have been resolved; (ii) fungi display a wide range of life cycles, geographical and diverse ecological systems as their habitats encourage them to adapt to new environments, thus allowing speciation processes; and (iii) numerous species complexes are known among common plant pathogenic genera. With fungi such as *Colletotrichum* and *Cercospora*, the morphological distinctions among them are at best arbitrary, and have often been named depending on the host, which is ridiculed sometimes as 'a parrot on a mango tree'. Some species complexes have been lumped together again on morphological similarities. The current botanical nomenclature does not recognize asexual entities as familiar, convenient names to pathologists even when sexual stages are definitely known. 'The ecological species concept' and 'ecological speciation' have been proposed as an important component speciation among living organisms such as birds among neighbouring islands (Darwin and Wallace, 1859); fish (Hatfield and Schluter, 1999); lizards between sub-humid and wet areas (Ogden and Thorpe, 2002; Richmond and Reeder, 2002); and insects (Via *et al*., 2000; Rundle and Nosil, 2005; Barat *et al*., 2008).

Ecological speciation in fungi has been reported and/or genetically examined (Chase and Ullrich, 1990; Antonovics *et al*., 2002; Couch *et al*., 2005; Fisher *et al*., 2005; Lopez-Villavicencio *et al*., 2005) or evidenced to be of low genetic diversity (Ahrén *et al*., 2004). Jeewon and Hyde (2007) observed that most of the molecular techniques cannot discriminate between active and inactive stages of fungi because mycelial propagules or dormant spores can be numerically dominant populations but in their natural environment they may be functionally insignificant. Among various molecular techniques tried in fungal diversity studies, polymerase chain reaction (PCR)-based fingerprinting and oligonucleotide fingerprinting of ribosomal RNA gene (ORFG), arrayed rRNA gene clones into taxonomic clusters through a series of hybridization experiments (Kirk *et al*., 2004) are promising ones. The most frequently used methods in assessing fungal diversity are denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) (Li *et al*., 2005; Raberg *et al*., 2005; Wakelin *et al*., 2008b). However, there has been no study of diversity of NTF/NPF using molecular methods.

4.2.2 Taxonomy

Taxonomy (*taxon* = order or arrangement, *nomos* = law) is defined as 'a study at producing a system of classification of organisms, which best reflects the totality of their similarities and differences'. It includes taxonomy *sensu stricto*, i.e. the theory of classification, systematics (science of diversity of organisms) and identification or correct assignment to a universally acceptable diagnosis. Species concept is not uniform throughout the living world. For instance, species concept is briefly described here in relation to nematodes – plant-parasitic nematodes in particular. From an assortment of diverse individuals belonging to a community of identical or non-identical populations, the phenomenon is important for identification, i.e. to fix the *phenon* (pl. *phena*) to one or more species. Two concepts of species are relevant in nematode taxonomy: biological species and parthenogenetic or thelytokous species. Genetics of speciation in fungi is not well known. Four or five genes were found to be involved in the inter-sterility among *Heterobasidion* species (Chase and Ullrich, 1990). DNA multi-locus typing showed that different

clones of fungus *Heterobasidion annosum* are associated with different environments (Fisher *et al*., 2005). The adaptation to new environments limits the organism's ability to successfully disperse in nature. The population structure in asexual parasites reflects host or habitat adaptation at all loci as the selection at one locus results in hitchhiking of the whole gene (Giraud *et al*., 2008). Biology is not possible without taxonomy. Taxonomy has a great predictive value because the constituent members are largely similar, which is needed for any branch of biology.

4.2.3 Biological species

A biological species is defined as 'an independent reality which can be identified by a given population statistics'. A unified concept of species is not possible to achieve because those working with different groups have to accept the reality of the situation in the group with which they work. It must have three identities: (i) a reproductive community; (ii) an ecological unit; and (iii) a genetic unit. Not all the nematodes conform to all these criteria. The subspecies is the only intra-specific taxonomic category recognized by the International Code of Zoological Nomenclature (ICZN). A subspecies is in fact a geographical or ecological population. A subspecies is recognized on the basis of the 75% rule, being the 75% disagreement between two species. A polytypic species comprises several subspecies, while a monotypic taxon at any level represents only a single immediately subordinate taxon.

4.2.4 Parthenogenetic or thelytokous species

Although generally bisexual, quite a number of nematode species are parthenogenetic. Thelytokous species represent the smallest groups that are consistently and persistently distinct and distinguishable. These species may also be called morphological or phenetic species, being a morphological identity. Many fungi are also phenetic. Even DNA sequencing or other molecular genetic methods have not solved the problems of identity or diagnosis. Their classification remains arbitrary and far from natural or evolutionary. Geographical speciation is due to reproductive isolation, in which gene flow has been restricted to the population in a geographic community. Among fungi, obligate parasites have fewer hosts and still fewer subspecies. Plant and veterinary nematode parasites also have restricted host range, although with exceptions; *Plasmodiophora brassicae* Woronin, which is an obligate plant pathogen, has a very wide host range and is monotypic from the class to the species status.

4.2.5 Taxonomy of nematophagous fungi

The taxonomic position of some of the NF has been clarified with the discovery of the sexual stages, as the teleomorphs of the fungi (Pfister, 1997), such as of *Arthrobotrys*, *Monacrosporium* and *Dactylella* have been identified as *Orbilia* spp., belonging to the Discomycetes (Ascomycetes). *Nematoctonus* spp. are different from all other nematode-trapping Deuteromycetes, not only by being both nematode-trapping and endoparasitic but respective nematode hosts. Moosavi and Zare (2012) briefly described the taxonomy of NF and considered teleomorphs of most nematode-trapping species, and their types of trapping devices to be useful to determine taxonomic position (Ahrén *et al*., 1998). Further, Scholler *et al.* (1999) attempted to classify NF based on their genetic data of *Arthrobotrys* (adhesive three-dimensional networks), *Dactylellina* (stalked adhesive knobs and/or non-constricting rings), *Drechslerella* (constricting rings) and *Gamsylella* (adhesive branches and unstalked knobs).

As defined by Durrieu (1970), a proto-homotypic parasite/pathogen has the same distribution as that of the host, narrow or wide (Dasgupta and Mandal, 1989). Further, more eu-homotypic (a parasite with its host) parasites tend to reach an optimum balance with their host. Consequently, a disaster becomes less and less likely. This is relevant in using NPF in biological control. Natural control has no problem with the combination between parasitic nematodes and their respective

hyperparasitic fungi. But their application in biological control is not likely to be very successful because the hyperparasitic fungi may not eradicate the parasitic nematode. Hence, biological management is likely to be more effective.

4.2.6 Classification

Classification (= generic classification) is primarily based on conidial characteristics, i.e. conidial size, septation, and type of conidiogenous cells (Oudemans, 1885; Subramanian, 1964). Identification of species *per se* become easier but the genera remain difficult to distinguish morphologically alone. Since 1930, NTF have been mostly described under *Arthrobotrys* Corda, *Dactylaria* Sacc. and *Dactylella* Grove. Recent classification based on rDNA sequence has retained those with constricting rings to *Arthrobotrys*, which may realistically contain 63 species (Kirk *et al.*, 2008). Oudemans (1885) introduced two species of *Monoacrosporium*. The genus *Gamsylella* created by Scholler *et al*. (1999) was not considered as valid, and was later included in *Dactylellina* Morelet 1968 with *D. leptospora* (Drechsler) as the type species. Yet the genus status of *Gamsylella* remains dubious. Subramanian (1964) described *Drechslerella* based on the type species, *D. acrochaeta* (Drechsler) Subram. Species Fungorum (2014) includes 273 records as anamorphic *Orbilia* (family Orbiliaceae, order Orbiliales). Li *et al*. (2005) transferred these species to *Arthrobotrys* and Yang and Liu (2006) combined this genus with *Dactylellina* based on molecular phylogenetic analysis. Rubner (1996) revised the generic concept of *Dactylella* and excluded the nematode-trapping species. While 108 *Dactylella* species are included in Index Fungorum (2014), Kirk *et al*. (2008) estimated only 62 species. NF comprise more than 200 species of taxonomically diverse fungi that are able to attack living nematodes (juveniles, adults and eggs). *Arthrobotrys oligospora* Fresen was the first fungus to be reported in this group in 1852, although its predatory nature was first observed by Zopf (1888).

4.2.7 Molecular genetics-based classification of trapping devices

Molecular phylogeny based on the sequences of 18S rDNA has yielded a phylogenetic tree for the NTF, where the phylogenetic pattern is based according to the structure of the trapping devices (Table 4.1) as mentioned in Nordbring-Hertz *et al*. (2006) and Ahrén *et al*. (1998). Durrieu (1970) reported trapping devices in fungi having having clamp connections, typical of basidiomycetes, and several species producing gilled mushrooms as their fruit-bodies (*Hehenbuehelia* spp.).

4.2.8 Molecular phylogeny in evolution

There is a growing body of evidence that the parasitic habit of NTF has evolved among cellulolytic and lignolytic fungi as a response to nutrient deficiencies in nitrogen-limiting habitats (Barron, 1992).

4.2.9 Phylogenetic significance and evolution of trapping devices

While morphologic classification of fungi is inadequate, molecular phylogenesis has been in use for over a decade in fungi to establish their natural or phylogenetic relationships and to evaluate the phylogenetic significance of different morphological characters. Cai *et al*. (2009) first showed the phylogenetic significance of molecular data on NTF. Rubner (1996) first used molecular phylogenetic analysis of trapping devices to rationalize the classification of NTF. Later, rDNA sequencing has been used by several groups (Swe *et al*. 2011). For example, Ahrén *et al*. (1998) showed that NTF have evolved from three lineages based on different types of trapping devices. Scholler *et al*. (1999) classified NTF into four genera based on 18S, ITS r-DNA analysis. Li *et al*. (2005) re-evaluated 28S, 5.8S and β-tubulin analyses but the genus *Gamsylella* was not accepted. Yang *et al*. (2007b) traced the evolution of trapping devices in predaceous fungi based on analyses of ITS, rpb2, ef-1 and β-tubulin sequences. The relationship of the genus

Table 4.1. Species of nematophagous fungi and their trapping devices/mode of infection.

Trapping device/mode of infection	Fungi	Taxonomic classification
Adhesive nets	*Arthrobotrys oligospora*	Deuteromycetes
	A. conoides	
	A. musiformis	
	A. superba	
	Duddingtonia flagrans	
Adhesive branches	*Monacrosporium gephyropagani*	Deuteromycetes
Adhesive knobs	*M. ellipsosporum*	Deuteromycetes
	M. haplotylum	
Constricting rings	*A. dactyloides*	Deuteromycetes
	A. brochopaga	
Snares and adhesive knobs	*Dactylella ellipsospora*	Discomycetes Ascomycetes
Adhesive spores	*Nematoctonus concurrens*	Basidiomycetes
	N. leiosporus	
	Drechmeria coniospora	
	Hirsutella rhossiliensis	Deuteromycetes
Ingested spores	*Harposporium anguillulae*	Deuteromycetes
Zoospores	*Catenaria anguillulae*	Chytridiomycetes
	Haptoglossa dickii	Oomycetes
Adhesive hyphae	*Stylopage hadra*	Zygomycetes
	Cystopage cladospora	
Toxic droplets	*Pleurotus ostreatus*	Basidiomycetes
Appressoria	*Pochonia chlamydosporia*	Deuteromycetes

Gamsylella to *Arthrobacter* and *Dactylella* remained unresolved. Phylogenetic analysis of nucleotide sequences mentions the two kinds of early trapping devices – adhesive traps and constricting rings (Li *et al*., 2005; Yang *et al*., 2007a). Smith and Jaffe (2009) developed Orbiliales ITS and 28S rDNA-specific PCR primers that directly detect NTF without culturing.

4.2.10 Ancient nematode-trapping fungi

The fossil *Palaeoanellus dimorphus* lived 100 MYA (million years ago) in a limnetic-terrestrial microhabitat (Schmidt *et al*., 2008). The fossil perhaps represents the anamorph of an ascomycete with unicellular hyphal rings as trapping devices and formed blastospores, which turned into a yeast stage. A predatory form of fungi with a yeast stage is now extinct. Fossils of *Oligaphelenchoides atrebora* found in a piece of Mexican amber approximately 25 million years old were found to be parasitized by fungi showing a striking resemblance to the present day nematophagous species (Jansson, 1986).

4.2.11 Diversity in fungi – nematophagous fungi in particular

Subramanian (1964) estimated that there are about 1 million fungi of which 70,000 have been described. About 205 new genera have been described by Subramanian from University of Madras, India. Of the 27,000 species from India, 205 colonize diversified habitats (Sarbhoy *et al*., 1996). Hawksworth (1991) estimated that there are 1.5 million global species of fungi of which 1 million have been described. Hyde (2001) observed most of the undescribed fungi are micro-fungi belonging to poorly investigated areas and less explored niches, substrates, hosts and habitats. There are several authors who have claimed otherwise (see Swe *et al*., 2011), all of whom agree that wide differences exist between those 'guesstimated' and those that have been described.

On the contrary, Swe *et al*. (2011) observed that since 2001, there has been a relatively large number of studies on fungal diversity particularly under extreme environmental conditions: Antarctica (Connell *et al*., 2006, 2008; Fell *et al*., 2006) and desert grasses (Porras-Alfaro *et al*., 2008); marine (Hyde, 1996; Poon and Hyde, 1998; Barata, 2006; Hyde and Sarma 2006; Lai *et al*., 2007; Laurin *et al*., 2008; Nambiar *et al*., 2008) and freshwater habitats (Cai *et al*., 2003; Tsui *et al*., 2003; Fryer *et al*., 2004; Tsui and Hyde, 2004; Duarte *et al*., 2006; Sole *et al*., 2008); terrestrial habitats (Hyde and Alias, 2000; Sun and Liu, 2008; Wakelin *et al*., 2008a); environmentally polluted habitats (Indra and Meiyalagan, 2005; Zafar and Ahmad, 2005; Ellis *et al*., 2007; Duarte *et al*., 2008; Stefanowicz *et al*., 2008; Turnau *et al*., 2008); decaying litter (Tsui *et al*., 2000; Cai *et al*., 2003; Tsui and Hyde, 2004; Gulis *et al*., 2008; Lonsdale *et al*., 2008); and dung (Juniper, 1953, 1954, 1957). Ahrén *et al*. (2004) showed that genetic diversity is limited in NTF, such as among the isolates of the single species *Duddingtonia flagrans*, which has demonstrated worldwide dispersion. More than 50 species of predaceous Hyphomycetes have been recorded from aquatic habitats (Ingold, 1944; Peach, 1950, 1952, 1954; Anastasiou, 1964; Hao *et al*., 2004, 2005). *Arthrobotrys dactyloides* Drechsler was the first species reported from brackish water (Johnson and Autery, 1961). Swe *et al*. (2008a,b) recorded several species from mangroves. Ecological surveys indicate their occurrence in all types of climates and habitats across the world (Gray, 1987).

There have been relatively few diversity studies of NTF (aquatic habitats: Hao *et al*., 2005; heavy metal-polluted soils: Mo *et al*., 2006, 2008). Mo *et al*. (2008) found that the diversity index of NTF was positively correlated with concentration of heavy metals. This finding corroborates that an extreme environment generally leads to adaptation, ecological speciation, or eradication of the species from the given habitat.

Speciation is possible due to geographic and reproductive isolation. Speciation is stimulated by unfamiliar habitat to the organism. Geographical distribution increases with speciation – ecological speciation in particular.

In a few instances, why a given species remains restricted to its older habitat is often difficult to explain. *Meloidogyne brevicauda*, which causes pinhole pit gall in mature tea, is considered the third most important among eight *Meloidogyne* spp. infecting tea. *M. brevicauda* is literally biphagous but virtually monophagous as it occurs on saffron (*Crocus sativus*) as well as on tea in Apsheron in Azerbaijan (Kasimova and Atakishieva, 1980). *M. brevicauda* is a pest in the tropics for tea in South India, but in north European Azerbaijan (40.8°N) on saffron, two land races are suggested. It is strange that while Azerbaijan is surrounded by Georgia and Armenia in the north-west, Russia (in the then USSR) in the south-east and Iran in the south-west, *M. brevicauda* has not spread to tea in this region. *M. brevicauda* has been found as an endemic pest in three single states of India in a single jungle (the Periyar) area, one each at Coonor in the Nilgiris (Tamil Nadu), Wynad district (Kerala) and Karnataka (Venkata Ram, 1963; Muraleedharan and Selvasundaram, 2001). The ecological significance of the jungle in either case is intriguing.

Fungal biodiversity in respect of distribution, conservation and prospecting of fungi from India has been reviewed (Monoharachary *et al*., 2005). The number of fungi recorded from India exceeds 27,000 species of which 205 genera have been described by Subramanian from diversified habitats, which indicates a ten-fold increase in the last 70 years (Sarbhoy *et al*., 1996), the largest biotic community after insects. The kingdom Eucaryota has four phyla, 103 orders, 484 families and 4979 genera described between 1905 and 1995, which includes: Myxomycetes (World:India: 450:380); Mastigomycotina (308:205), Zygomycotina (55:50); Ascomycotina (2000:745); Basidiomycotina (357:232); Deuteromycotina (4100:468); total (7270:2080). Hawksworth *et al*. (1995) recognized 11 phyla. Manoharachary and his co-workers have added 12 new genera, 60 new taxa and 500 new additions to the fungi of India. The existence of a fossil record dates back to the early Phanerozoic and into the Proterozoic geological era (Pirozynski and Hawksworth, 1988).

4.2.12 Diversity of toxin-producing nematophagous fungi/nematode-trapping fungi in India

Four species of NF from North India (Das Gupta *et al*., 1964; Das Gupta and Shome, 1966), one species from Assam (Walia and Swarup, 1985), several (up to 31) species from Delhi soils (Sachchidananda and Swarup, 1966a,b, 1967a,b; Walia and Swarup, 1985; Srivastava, 1986; Gupta *et al*., 1988; Mittal *et al*., 1989), three from Bihar (Srivastava and Askary, 2000), 31 from Uttar Pradesh (Saxena and Mukerji, 1991) and ten NTF from agriculture soils (Sachchidananda, 1965; Sacchidananda and Ramakrishnan, 1971), two each from Rajasthan and Gujarat (Sanyal, 2000), one from Karnataka (Nagesh *et al*., 2005), 13 species of NF from Maharashtra (Patil and Pendse, 1981) and five from Tamil Nadu (Rajeswari and Sivakumar, 1999) have been recorded. Singh *et al*. (2005) conducted surveys in eastern Uttar Pradesh (UP), India and reported the distribution of predaceous fungi in agriculture soils, whereas in rhizospheric soils of UP, the occurrence of NF was reported by different research workers (Saxena and Mukerji, 1991; Singh *et al*., 2007). The colonization of these fungi was studied by Kumar *et al*. (2010) in different substrates. In 1967, Singh reported *Catenaria vermicola* from India. Gupta *et al*. (2003) conducted studies on the nematode parasitism by *C. anguillulae* in the rhizosphere of some fruit plants.

Diversity of NPF has not been widely studied in India. *Catenaria anguillulae* (Ca) was known in India from 12 states at 39 locations, chiefly UP (Vaish and Singh, 2002). Of 490 soil samples, 451 yielded Ca remaining present throughout the year, irrespective of soil types and crops grown. In some districts of UP, Singh *et al.* (2006) identified the following NPF species (respective percentage frequency in parenthesis) comprising *A. oligospora* (18.51%), *A. dactyloides*, *A. conoides*, *D. brachopaga* (10.49%), *Monacrosporium eudermatum* (12.96%), *Monacrosporium parvicolis* and *Stylopage hadra*, whereas the endosporic fungi comprising *C. anguillulae*, *Harposporium anguillulae* (having least frequency among the fungi encountered – 4.32%) and *Meria coniospora* were encountered. Higher frequency was found in rhizosphere soils which were regularly amended with farmyard manure (FYM) and other organic matter as well as severely infested with nematodes. Nematode frequencies varied among fields, locations, nematode populations and years.

4.3 Distribution

Distribution may refer to global, continental, regional, local and field occurrence and intensity of organisms. Spatial distribution and vertical distribution are generally excluded from this article as we intend to restrict to geographical distribution. Geophytonematology (Dasgupta, 1998) refers to pathogeography of a nematode(s). Climate is the most important composite factor that influences geographical distribution. The most important climatic factor for any organism's geographical distribution is its preferable temperature regime in the prevailing temperature regime. Temperature is the driving variable in any kind of distribution of any organism. Moisture is probably nearly as important, which is manifested by other kinds of influences like seasonal variations but becomes rarely critical in agricultural soils. Regional distribution of NF is also affected by the distribution of their respective hosts. Parallel evolution or co-evolution determines, among others, the geographical distribution of both the host and its respective parasites. Temperature optima range for both the nematode and the NF determine the ideal geographic distribution of both. Apparently, there are optimum temperature bands in two hemispheres and a true tropical species is likely to have a contiguous band without being separated at the equator. The tropical lowland forest is the most equitable climate providing optimum temperature and moisture regimes for plants, parasitic nematodes and their respective nematode-destroying fungi (Dasgupta, 1998).

Soil and plant-parasitic nematodes are generalists rather than r- or K-selection strategists. The respective species are called r-strategists and K-strategists. The r is the intrinsic growth rate of a species and the K is the asymptote – a line approaching a curve, but never reaching it within a finite distance.

They show moderate diversity and low temperature density. An r-selection strategist depends on its faster rate of growth for its survival. A K-selection strategist depends on a high initial population and maintains its survival with a low growth rate. With increasing climatic or other stresses, the nematodes have both diversity and density resulting in their high adaptability only second to arthropods (Dasgupta, 1998). Correspondingly, the fungi that act as nematode-destroying fungi are mostly mesophilic (these organisms prefer moderate temperature range), except those in extreme regions like the Antarctica and the deserts or at high altitudes as in the temperate and the subtropical hills. Thus, altitude becomes an important factor at least in certain geographical regions. Nematode species of the same genus have different patterns of geographical distribution. For example, in *Meloidogyne*, *M. hapla* prefers low temperature while *M. incognita*, *M. javanica* (prefers extreme wet and dry conditions too) and *M. arenaria* prefer a warm climate. These three species have widest, wider and more restricted distribution and host range, respectively. It is likely that their respective nematode-destroying fungi would also reflect similar preferences. Similarly, the cyst nematodes generally prefer cooler climates except *Heterodera cajani*, which prefers warmer tropics such as parts of India.

Plant-parasitic nematodes and their respective nematode-destroying fungi can also adapt to a climate that may not be exactly the same as their earliest homelands. Once established inside its host, a plant-parasitic nematode is safe from the outside environment. Table 4.2 partially depicts the distribution of NF in almost all habitats and climatic conditions of the world. The distribution of different NF is proto-homotypic, the distribution of host and parasite/pathogen are as limited as that of the host.

4.4 Factors Affecting the Distribution of Nematode-trapping Fungi

This section draws heavily from Swe *et al*. (2011). The distribution and occurrence of NPF with species and groups of fungi is associated with specific soil variables, in particular pH, moisture, nutrients (N,P,K), heavy metal and nematode density (*Gardner and Pillai, 1986 – on a superparasite of the mosquito *Aedes australis*; *Gray, 1985, *Gronvold *et al*., 1999 – *Cooperia oncophora* larvae; Persson and Baath, 1992; *Jaffee, 2004; *Jaffee, 2006; Mo *et al*., 2008; *Sánchez-Moreno *et al*., 2008; those marked with * indicate interaction between ecological factors acting on NTF on plant-parasitic nematodes, which is commonly observed in any ecosystem). There was a positive correlation of N, P and K with nematode density. Isolation of the species bearing stalked (= Duddington's snare) knobbed trapping devices and constricting rings (*Drechslerella*) were more readily found from richer soils where nematode density was higher. However, those species having net-forming devices (*Arthrobotrys*) have been found mostly independent of soil fertility, especially low K (Burges and Raw, 1967). The distribution of NTF was affected by heavy metal concentrations and was positively correlated with lead concentrations (Mo *et al*., 2006, 2008). Ethylene diamine tetra-acetic acid (EDTA) treatment has been found to detect various stages of fungi in aggregates in the sediments from –5000 m in deep sea (Damare and Raghukumar, 2008). In that case, it may be used as an indicator for the occurrence of NPF in terrestrial and aquatic and marine habitats. The soil variables such as soil bacteria, fungi, nematodes and NPF vary with soil depth (Mankau and McKenny, 1976). Gray and Bailey (1985) found NTF with constricting rings, adhesive branches and adhesive knobs being restricted to the upper litter and humus layer while the net-forming predators and endoparasites are distributed at all depths tested in deciduous woodland although significantly located in the lower depths (McSorley *et al*., 2006) and a high level of nematode-trapping activity has been recorded from the rhizosphere (Peterson and Katznelson, 1964; Mitsui *et al*., 1976; Persmark and Jansson, 1997; Wang *et al*., 2003; McSorley *et al*., 2006). The predators forming spontaneous traps are restricted to the organic soils of the hemi-edaphic zone rich in nematodes. NPF are small enough to be affected by microclimates within the soil (Gray, 1985). However, the exact populations vary largely with plant and soil types (Jansson and

Table 4.2. Distribution of nematophagous fungi in different habitats.

Country/region	Nematophagous fungi	Habitat	Reference
Antarctic	*Arthrobotrys robusta* *Dactylella cionopaga* *D. gephyropaga* *D. ellipsospora* *D. phymatopaga* *D. leptospora* *D. stenobrocha* *Dactylaria gracilis*	Soil and vegetation	Gray (1982, 1983, 1985); Gray and Smith (1984)
Austria	*Arthrobotrys conoides* *A. dactyloides* *A. pauca* *A. oligospora* *Dactylaria thaumasia* *Dactylella* sp. *Dactylella brochopaga* *Harposporium anguillulae* *Monacrosporium ellipsosporum* *M. gephyropagum* *M. psychrophilum* *M. robustum*	Soil	Plenk (1987)
Australia	*Arthrobotrys* spp. *A. amerospora* *A. eudermata* *A. megalospora* *A. musiformis* *A. oligospora* *A. thaumasia* *A. conoides* *Dactylellina ellipsospora* *D. haptotyla* *Dactylellina* cf. *leptospora* *Dactylellina* spp. *Drechslerella dactyoides* *Duddingtonia flagrans* *Gamsylella* sp. *Gamsylella arcuata* *G. arcuata* *G. gephyropaga* *G. robusta*	Wood chips, cultivated soils, organic matter, moss cushions and fresh dung of livestock	McCulloch (1977); Faedo *et al.* (1997); Park *et al*. (2002)
Azerbaijan	*Arthrobotrys chazarica* *Nematophagus tabrizicus* *Nematoctonus haptocladus* *N. concurrens* *N. leiosporus* *N. leptosporus*	Soil	Mekhtieva *et al*. (1984); Mekhtieva (1998)

Continued

Table 4.2. Continued.

Country/region	Nematophagous fungi	Habitat	Reference
Brazil	*Arthrobotrys conoides* *A. fusiformis* *A. oligospora* *A. oviformis* *A. robusta* *A. thaumasia* *A. musiformis* *Botryotrichum nematophagus* *Monacrosporium* sp. *Monacrosporium eudermatum* *M. drechsleri* *M. ellipsosporum* *M. gephyropagum* *M. parvicollis*	Field soils, crop roots	Naves and Campos (1991); Pria *et al.* (1991); dos Santos *et al*. (1991, 1993); Dias *et al*. (1995)
Canada	*Arthrobotrys oligospora*		Estey and Olthof (1965)
China	*Arthrobotrys lacdodes* *A. musiformis* *A. venusta* *A. vermicola* *A. guizhouensis* *A. oligospora* *Dactylella atractoides* *D. stenomeces* *D. zhongdianensis* *Monacrosporium acrochaetum* *M. heterobrochum* *M. indicum* *M. longiphorum* *M. megalosporum* *M. mutabile* *M. phymatopagum* *M. lysipaga* *Monacrosporium* spp. *M. thaumasium* *Stylopage araea* *S. leiohypha* *Tridentaria* sp. *Verticillium balanoides*	Agricultural field soils, root-knots, mountains, aquatic environments	Liu *et al*. (1992); Lei *et al.* (1994); Zhang *et al*. (1994, 2005); Gao *et al.* (1996); Mo *et al*. (2001); Hao *et al*. (2005)
Costa Rica, Nicaragua, Panama	*Arthrobotrys conoides* *A. musiformis* *A. oligospora* *A. oviformis* *A. krigizica* *Acaulopage pectospora* *D. leptospora* *Dactylella sclerohypa* *Monacrosporium eudermatum* *M. gephyropagum* *M. haptotylum* *M. parvicollis* *M. candidatum* *Stylopage* sp.	Arable soils	Persmark *et al*. (1995)

Continued

Table 4.2. Continued.

Country/region	Nematophagous fungi	Habitat	Reference
Denmark	*Arthrobotrys dactyloides* *Dactylaria psychrophila*	Soil, rotting leaves and leaf mould	Shepherd (1956)
Ecuador	*Monacrosporium* spp. *M. parvicolle*	Soils	Rubner (1994)
El Salvador	*Stylopage hadra* *Dactylella*	Soils	Bucaro (1983)
Fiji	*Arthrobotrys musiformis* *A. oligospora* *A. cladodes*	Fresh dung of small ruminants	Manueli *et al*. (1999)
France	*Arthrobotrys irregularis* *A. conoides*		Cayrol (1988)
India			
Assam	*Dactylaria thaumasia*	Soil	Walia and Swarup (1985)
Bihar	*Cystopage cladospora* *Haprosporium arcuatum* *Monacrosporium megalosporum* *Stylopage leiohypha*	Agricultural soils	Srivastava and Askary (2000)
Delhi	*Arthrobotrys conoides* *Arthrobotrys* *Cystopage* *Cy. lateralis* *C. intercalaries* *Dactylaria psychrophila* *D. brochopaga* *Dactylaria* *Monacrosporium cystosporum* *M. gephyrophagum* *M. megalosporum* *M. salinum* *Monacrosporium* *Myzocytium lagenidium* *Nematoctonus* *Stylopage hadra* *S. grandis* *S. leiohypha*	Wheat seed galls, soils	Sachchidananda and Swarup (1966a,b, 1967a,b); Walia and Swarup (1985); Srivastava (1986); Gupta *et al*. (1988); Mittal *et al.* (1989)
Karnataka	*Arthrobotrys oligospora* *A. cladodes* *Dactylaria* sp. *Laginidium* sp. *Monacrosporium gephyrophagum*	Field soils, and faecal matter of goat, sheep and cattle in open grazing fields	Gowda *et al*. (1982); Nagesh *et al*. (2005)
Gujarat and Rajasthan	*Arthrobotrys oligospora* *Duddingtonia flagrans*	Fresh faeces of ruminants	Sanyal (2000)
UP/India	*Acaulopage pectospora* *Arthrobotrys conoides* *A. dactyloides* *A. oligospora* *Dactylaria brochopaga* *Monacrosporium eudermatum* *M. pervicollis* *Stylopage hadra*	Rhizospheric soil, cultivated lands, rotting wood, etc.	Das Gupta and Shome (1966); Saxena and Mukerji (1991); Singh *et al*. (2001, 2006); Kumar *et al*. (2010)

Continued

Table 4.2. Continued.

Country/region	Nematophagous fungi	Habitat	Reference
Maharashtra	*Harposporium crassum* *Stachybotrys atra*	Soil	Patil and Pendse (1974)
Tamil Nadu	*Arthrobotrys cladodes* var. *macroides* *A. oligospora* *Arthrobotrys* sp. *Dactylella brochopaga* *Dactylaria thaumasia*	Soils	Rajeswari and Sivakumar (1999)
Iraq	*Arthrobotrys* *Cystopage* *Dactylella* *Dactylaria* *Monacrosporium* *Stylopage*	Soils	Muhsin and Kasim (1998)
Iran	*Arthrobotrys oligospora* *Duddingtonia flagrans* *Haptocillium sphaerosporum*	Sheep faeces	Bahadori *et al.* (2004)
Ireland	*Acaulopage pectospora* *Dactylella cionopaga* *D. phymatopaga* *D. brochopaga* *D. mammillata* *D. psychrophila* *Cephalosporium balanoides* *Trichothecium cystosporium*	Soils	Gray and Bailey (1985)
Kenya	*Acrostalagums obovatus* *Arthrobotrys dactyloides* *A. oligospora* *A. superba* *Dactyllela lobata* *Haptoglosa heterospora* *Harposporium* sp. *Ha. anguillulae* *Monacrosporium cionopagum* *Nematoctonus georgenious*	Natural forest, shrubs, vegetables, napier grass and maize/bean intercrop	Wachira *et al.* (2010)
Korea	*Arthrobotrys oligospora* *A. conoides* *A. arthrobotryoides* *M. ellipsosporum* *M. thaumasium* *Dactylellina candidum* *Dactylella lobata*	Uplands, greenhouse and mountains	Jeong and Kim (1988); Kim *et al*. (2001); Lee *et al*. (2003); Wu *et al*. (2012)
Oman	*Arthrobotrys oligospora* *A. multiformis* [=*Monacrosporium multiforme*] *A. oudemansii* [=*Monacrosporium elegans*] *A. javanica* *Drechslerella brochopaga* [=*Arthrobotrys brocophaga*] *Gamsylella gephyropaga* [=*Monacrosporium gephyrophagum*]	Soil and leaf litter	Elshafie *et al*. (2003)

Continued

Table 4.2. Continued.

Country/region	Nematophagous fungi	Habitat	Reference
Mexico	*Arthrobotrys* *Dactylella*	Semi-arid soils	Olivares *et al*. (2002)
New Zealand	*Arthrobotrys oligospora* *A. brochopaga* *A. cladodes* *A. conoides* *Monacrosporium haptotylum* *M. gephyropagum*	Sheep faeces, pasture	Hay *et al.* (2002)
Philippines	*Arthrobotrys cladodes*		Villanueva and Davide (1984)
Russia	*Arthrobotrys oligospora* *A. globospora*		Matskevich (1990); Matskevich *et al*. (1990, 1991)
Saudi Arabia	*Arthrobotrys candida* *A. dactyloides* *A. musiformis* *A. oligospora* *Dactylaria brochopaga* *D. candida* *D. ellipsospora* *Monacrosporium cionospagum* *M. phymatopagum* *Stylopage lepte*	Cultivated soils	Saadabi (2006)
Scotland	*Arthrobotrys arthrobotryoides* *A. conoides* *A. eudermata* *A. gephyropaga* *A. kirghizica* *A. oligospora* var. *oligospora* *A. salina* *A. superb* *A. musiformis* *A. oviformis* *A. robusta* *Dactylellina ellipsospora* *Da. phymatopaga* *Drechslerella anchonia* *Dr. brochopaga* *Stylopage grandis* *S. hadra* *S. leiohypha*	Soils	Boag and Lopez-Llorca (1989); Saxena (2008)
Spain	*Arthrobotrys oligospora* var. *oligospora* *A. oligospora* var. *microspora* *A. oligospora* var. *sarmatica* *A. javanica* *A. arthrobotryoides* *A. brochopaga* *A. musiformis*	Forest and crop fields	Bernabeu *et al.* (2003)

Continued

Table 4.2. Continued.

Country/region	Nematophagous fungi	Habitat	Reference
South Africa	*Duddingtonia flagrans* *Acremonium* *Arthrobotrys conoides* *A. dactyloides* *Cystopage* sp. *Cystopage cladospora* *Dactylaria brochopaga* *Haptoglossa heterospora* *Harposporium* sp. *Monacrosporium* sp. *M. gephyrophagum* *Meristacrum asterospermum* *Myzocytium* spp. *Stylopage leiohypha*	Leaf litter, soil, faeces from domestic and game animals, compost, groundnut field soils	Jones *et al*. (1996); Durand *et al*. (2005)
Sweden	*Arthrobotrys oligospora* *A. musiformis* *A. robusta* *A. superb* *A. cionopagum* *Dactylaria candida* *Genicukfera perpasta* *Monacrosporium psychrophilum* *Stylopage* sp.	Crop rhizosphere and agricultural soils	Persmark *et al*. (1996); Bordallo *et al*. (2002)
UK (Hampshire)	*Dactylella megalobrocha*		Glockling and Dick (1994)
USA	*Arthrobotrys conoides* *A. dactyloides* *A. haptotyla* *A. oligospora* *A. superb* *A. thaumasia* *Acrostalagmus obovatus* *Haptoglossa heterospora* *Harposporium anguillulae* *Macrobiotophthora vermicola* *Meristacrum* sp. *Me. asterospermum* *Monacrosporium* sp. *M. bembicodes* *M. eudermatum* *Nematoctonus leiosporus* *Stylopage* sp. *Stylopage hadra*	Agricultural soils, organic and conventional cropping systems	Paracer *et al*. (1966); Bernard and Arroyo (1990); Jaffee *et al.* (1998)

Lopez-Llorca, 2001). Peterson and Katznelson (1964) found that the greatest diversity of NTF, greater population density of NPF and root-knot nematodes occurred particularly in the upper 10–30 cm of groundnut soils. Nematode diversities were highest at a depth of 20 cm and nematode-trapping activity was not detected beyond 4 m in a freshwater pond (Hao *et al*., 2005). Spatial distribution has been extensively studied (Persson *et al*., 2000). Growth and dispersion of *Arthrobotrys superba* Corda has been studied under natural

conditions with a radioactive tracer technique (Segers *et al*., 2000; Minglian *et al*., 2004).

Major biotic and abiotic variables such as soil moisture, organic matter, pH, nematode density, soil nutrients (Gray, 1987) and submerged water condition on the distribution of NTF are not significant. Gronvold *et al*. (1999) obtained evidence of biotic and abiotic factors (and possibly their interaction) in the evolution of nematode-trapping devices in a nematode parasite on mosquito by the hyperparasite *C. oncophora* larvae. Effects of abiotic factors, such as temperature, pH, light, UV and nutrition on the trapping efficiency of NTF have been intensively studied (Ciordia and Bizzell, 1963; Gray, 1985, 1988; Morgan *et al*., 1997; Fernandez *et al*., 1999; Gronvold *et al*., 1999; Zucconi *et al*., 2002; Jaffee, 2006; Kumar and Singh, 2006a,b; Paraud *et al*., 2006; Sun and Liu, 2006; Gao *et al*., 2007).

4.4.1 Effect of crop and cropping systems

Plant species and cultivars affect soil microflora and fauna. Colonization, multiplication, survival and dissemination within and outside a habitat are largely governed by the interaction of crop, soil and fungi. Root exudates also influence the rhizospheric inhabitants including fungi (Vancura, 1988). Population densities of *A. dactyloides*, *A. superb* and *Monacrosporum ellipsosporum* were found greater in the rhizosphere of tomato root than root-free soil (Persson and Jansson, 1999). Limited studies have been undertaken on the impact of crop sequences on NF.

4.4.2 Effect of salinity

The effect of salinity on fungal growth has been studied in yeasts and moulds (Blomberg and Adler, 1993; Dan *et al*., 2002), marine fungi and mycorrhiza. (It may be noted that mycorhiza is the correct spelling – Gk. *myco* + *rhiza* – but it has gone into disuse, although Mycorhiza and Mycorrhiza have been alternatively used in older literature. Mycorrhiza has been used extensively for some decades. Nevertheless, we suspect a *lapsus calami* – a scientific error arising from a printing error (Dasgupta, 1988, 1998).) Some workers have studied the effects of salinity on the growth, mostly on the mosquito-parasitic fungi (Harrison and Jones, 1971; Lord and Roberts, 1985; Gardner and Pillai, 1986; Lord *et al*., 1988; Kramer, 1990; Teng *et al*., 2005) and fungi including some wood-rotting Basidiomycetes (Ritchie, 1959; Davidson, 1974; Byrne and Jones, 1975; Jones and Byrne, 1976; Kohlmeyer and Kohlmeyer, 1979; Siegel and Siegel, 1980; Hyde *et al*., 1987; Lorenz and Molitors, 1992; Clipson and Hooley, 1995; Akira and Tadyoshi, 1996; Castillo and Demoulin, 1997; Juniper and Abbott, 2006; Sharifi *et al*., 2007).

4.4.3 Effect of soil moisture and texture on transmission of the nematophagous fungus *Hirsutella rhossiliensis* to cyst and root-knot nematodes

Hirsutella rhossiliensis (Hr) produces non-motile spores as phialides, 30 µm long, that do not sporulate when submerged. Parasitism by Hr was greatest on the drier soils between irrigation furrows and greater than in wetter soil adjacent to irrigation furrows (Jaffee *et al*., 1989). Tedford *et al*. (1992) illustrated the moisture release curves, and the theoretical relationship between pore diameter, matric potential and drainage (Griffin, 1972). The non-motile spores of *H. rhossiliensis* adhere to vermiform nematodes present in the soil and thus initiate infection (Tedford *et al*., 1992). The second stage juvenile (J_2) of *Heterodera schachtii* and *M. javanica* was quantified in vials containing 17 cm^3 of loam, loamy sand, or sand without a host plant. Inoculation of fungus was done on day 0 as infected J_2 with sporulated fungus, and non-infected J_2 of either nematode were added after 14 days at 20°C and extraction was done 66 h later. Transmission by a J_2 which has acquired at least one spore to either nematode and in all three soils, had an inverse relationship to soil matric potential. Srivastava (1986) found some relationship between soil type and higher frequency of NF possessing a specific trapping device. Goswami and Rao (1995) indicated that soil texture and

structure influences nematode activity and growth of NF.

Duniway (1976) introduced this concept in *Phytophthora cryptogea* infecting safflower seedlings, sporangia formed in soil at the Ψm of −300 mb, and used to inoculate soils at higher Ψm ≥ −10 mb. When either sporangia or motile zoospores were used to inoculate soil, zoospores moved 25–35 mm in the surface water; in overflooded soils or through a coarse structured soil-mix the movement was reduced at Ψm ≥ Ψ −1 mb. The active movement of the zoospores in the soil-mix was reduced at Ψm ≤ −100 mb. The active movement in sieved and reconstituted silt loam and free sandy loam was limited to 5 mm at Ψm = −1 mb and was not detected at 1 mm in a clay loam at Ψm = −10 mb. This study shows the differential requirement for moisture for the production of sporangia, zoospores and their movements. Similarly, the moisture level or rather the moisture balance levels or rather the moisture potential values as governed by different soil textures and water balance in soil determine the movement of zoospores and, as a consequence, are likely to influence the epidemics in pythiaceous diseases. Similarly, the movement of nematode J_2s and NPF motile spores are likely to determine the increase or decrease of plant diseases due to these factors (Dasgupta, 1988).

Reduced transmission at high water potentials appeared to cause reduced fungal sporulation rather than reduced nematode movement. Transmission to *H. schachtii* (Hs) was greater or equal to transmission to *M. javanica.* The greatest transmission to either nematode was in loamy sand, intermediate in loam and the lowest in sand. The equation $P = 1 - tr \bullet col$ (where *col* = number of colonized nematodes added per vial at day 0 and tr = transmission parameter) described transmission to *M. javanica*. Previously, this equation was used to describe transmission of *H. rhossiliensis* to *H. schachtii*, to enable comparisons among experiments and also to help and make easier the incorporation of transmission data into epidemiological models (Anderson and May, 1981). Transmission to *Pratylenchus penetrans* in sand was greater in finer soils than in coarser soils, which was due to smaller pore size, facilitating the movement of vermiform and small nematodes (Jaffee *et al*., 1990). Transmission was greater to Hs than to *M. javanica*.

4.4.4 Comparison between organic and conventional cropping systems: interactive relationship among different factors

In order to test the three hypotheses ((i) population dynamics and number of species of NTF are greater in the plots in the organic CSs (cropping systems) than in the plots in the conventional CSs during the tomato growing season; (ii) organically managed soils are more suppressive to root-knot nematodes than are conventionally managed soils; and (iii) suppressiveness correlates with population densities of NTF (Jaffee *et al*., 1998) (see Table 4.1 for the nature of trapping mechanisms)), the nematodes and microbial biomass were quantified in organic and conventional CSs in the field plots at Davis, California in four replicates (0.135 ha/plot) for either management system. Plots were sampled three times each year for 2 successive years in 4-year CSs. The hypothesis that NTF would be more abundant in organically managed plots (Duddington, 1956) and were supported or inferred without relative quantification was partially supported; the number of NTF were slightly but significantly greater in organic than in conventional CSs. Two NTF species, *A. dactyloides* (Ad) and *Nematoctonus leiosporus* (Nl), were more frequent and denser in organic CS plots as compared to conventional CS plots. Other NTF species, *Arthrobotrys haptotyla* and *Arthrobotrys thaumasia*, tended to be more numerous in conventional than in organic plots. The total density of NTF was similar in organic and conventional plots. The more abundant were bacteriovorus nematodes. In organic plots, the microbial biomass (in terms of substrate-induced respiration) was greater than in conventional plots. Suppression of *M. javanica* was found positively related to microbial biomass and not to management system or population density of NTF, as measured in a bioassay. The results and discussion suggest the greater efficacy of the natural control than the abundance or frequency of a given plant parasite or hyperparasite.

In agricultural fields, results of the effect of organic amendments were varied in spite of partial quantification due to other deficiencies in experimental design and/or methodology (Dackman *et al*., 1992; Van den Boogert *et al*., 1994; Jaffee *et al*., 1996; Persmark, 1997); for other comments in detail, see Jaffee *et al*. (1998). With the help of two models it can be explained how organic amendments might stimulate NTF. The first one is a numerical response model, which presupposes that NTF are obligate parasites in nature and depend on nematodes for C, N and energy. Thus, organic amendments stimulate sequential increases in numbers of bacteria, bacteriovorous nematodes and NTF. Contrary to that, the supplemental N model presupposes that NTF are facultative parasites. By obtaining N from nematodes, the fungi can compete for C and energy bound in C-rich, N-poor plant litter. Jaffee *et al*. (1998) observed that this experiment is not designed to test these two models. Rather, they have emphasized quantification (and proper design and methodology as well). Their assumption is that the organic plots might contain greater number of bacteriovorous nematodes and NTF in organic CSs than in conventional CSs. They have determined SDs in addition to means to test the consistency of their data. They have also measured substrate-induced respiration on the basis of single replicates. Srivastava and Askary (2000) observed minimum correlation between frequency of NF and proportion of organic matter in soil. Wachira *et al*. (2010) found the influence of land use and soil management practices on the occurrence of nematode-destroying fungi in Kenya. Indra and Meiyalagan (2005) studied the diversity and distribution of microfungi in polluted and non-polluted water bodies from an industrial area of the River Palar, Vellore, India.

4.5 Definitive Methodology

4.5.1 New applications of statistical tools

Jaffee *et al.* (1998) described the definitive methodologies they have used for studying the factors of NTF. These methodologies merit wider adaptation. Researchers have so far used more of the statistics of mean, while the current stress is on the statistics of dispersion or spatial statistics. Analysis of variance based on the assumption of normality of distribution runs the risk of a wrong assumption. Therefore, we have used those data in a series that are distributed within ±1 SD, such as in the case of local macro-meteorological data for nearly 50 years. The statistics yield the most consistent data for the local area. Agro-meteorological trends in a location could be forecast on the basis that helps farmers in decision-making (Dasgupta, 2007; Mandal *et al*., 2009).

Garrett *et al*. (2004) have proposed certain statistical tools that can be applied in plant pathology. Those applicable to the geographical distribution in particular are listed here, and some relevant aspects. Such tools include survival analysis, nonparametric analysis of disease situations, multivariate analysis, neural networks, meta-analysis and Bayesian statistics. Garrett *et al*. (2004) also discuss one more important area: the caveats about inappropriate applications of statistical tools in plant pathology. Francl (2004) addresses the history, terminology and common misconceptions about artificial neural networks and provides recommendations. He also discusses applications of neural networks to leaf wetness estimation and infection prediction in wheat disease forecasting. Rosenberg *et al*. (2004) described the potential for meta-analysis in plant pathology such as its use in the response of yield to disease severity based on papers published in *Fungicide and Nematicide Tests.* They also suggest for authors how to make data more amenable to possible future use in meta-analysis. The assumptions of a statistic are crucial for its proper use. ANOVAs and regression analyses are well-known and frequently used for its convenience. But this is not always applicable in the contexts used by plant pathologists. Non-parametric approaches are often more appropriate to such situations. Turechek (2004) observes that non-parametric tools have unique uses where they are superior to parametric tests, or where no parametric approach exists, such as interspecific associations, co-occurrence of more than one plant disease in the same habitat and co-variance of

disease intensity for multiple diseases/pests on the same plant or in the same crop. The author describes an innovative nonparametric approach for testing the significance of the Jacquard index of association based on randomization methodology.

Using multiple regressions with least-square technique to minimize the number of multiple variables, a model was developed for yield loss in root-knot-infected tomato (Sen *et al*., 1983; Gilligan, 1986). This approach tended to solve the problem of autocorrelated variables – a vexing problem to biologists in general and plant pathologists in particular. Ultimately it could be reduced to only a single variable, i.e. plant height by using least-square technique (Schabenberger and Pierce, 2002). Gangwar *et al.* (1986a) used a method for analysis of modelling yield loss in indica rice in farmers' fields caused due to multiple pests. The weakness in the conclusion was its tentative nature owing to its lack of testing in comparative situations, which can always be done.

Scherm and Ojiambo (2004) observed that some popular statistical methods are inappropriate for such analysis while the survival analysis would be appropriate. They illustrated survival analysis to determine the timing of defoliation of blueberry leaves as a function of infection by *Septoria albopunctata*, and reference software for conducting this type of analysis.

4.5.2 Bayesian statistics

Bayesian statistics offers a different framework for statistical analysis by treating the parameter of interest not as a single value but rather as a random variable with a probability distribution. This is applicable in genomic analysis, disease mapping and experimental designs (Mila and Carriquiry, 2004).

4.5.3 Ordinal data

Plant pathologists measure disease by a scale of ordinal numbers – such as 0 (no disease) to 9 or 1 (no symptoms) to 4. At all stages they represent a limiting figure, such as 1 implies 'tending to 1'. Such data are discrete and the ordinal categorical random variable is amenable to Schabenberger and Pierce (2002). With sufficient observations, these data can be analysed with GLMs by assuming a multinomial distribution for the variable. A very useful alternative is using non-parametric analysis (Sprent and Smeeton, 2001; Turechek and Madden, 2003; Turechek, 2004). Non-parametric tests can be utilized for different types of analysis. A limitation is that until the previous 5 years or so, there was no satisfactory theoretical foundation of modelling data originating in factorial designs. We observed this while doing the experiments for five consecutive rice seasons (Gangwar and Dasgupta, 1983, 1984a,b; Gangwar *et al*., 1986c).

4.5.4 Linear mixed models

Garrett *et al*. (2004) discussed several other general statistical topics such as the application of discrete data using generalized linear mixed models (LMM), application of decision theory, model selection, and new applications in the context of microarray analysis.

4.5.5 ANOVA

ANOVA has been used for a long time and is still extensively used by plant pathologists for data analysis and inference (Gilligan, 1986). Since many response variables obtained by plant pathologists are discrete, they have adopted the easy solution by transforming the response of the dependent variable Y by transforming the data depending on the form in which they have been collected (disease incidence in per cent, counts of lesions or spores, colony-forming units (cfu per standard Petri plate)). This is of course a forced linearization of a non-linear data series; transformation had not been assumed for such purposes (Dasgupta and Chakraborti, 1990). They observed that biologists are required to study statistical techniques and often the respective assumptions are not valid for biological reality. Therefore statistics have to fit into biological reality rather than vice versa. Nevertheless, we are happy that some precise statistical methods have been developed to

solve such problems of biological reality (Verbeke and Molenbergs, 1997; Brunner and Puri, 2001; McCulloch and Searle, 2001). These advances promise to solve the problems more pragmatically than before.

4.5.6 Generalized linear mixed models

The models for continuous data are appropriate for discrete data but not vice versa. The biologists, macrometeorologists and pest (comprehensive sense) ecologists often use large number of variables; often a given set of data may not even contain sufficient data for a given variable. The occurrence of density in a location being zero used to baffle the investigator. The solution they take recourse to even now is transforming with (X +1). This is not reality though. Data collected over long periods or automatic constant recorders may bypass the problem, while taking ±1 SD as well as mean and selecting them out adds consistency to the selected data, which can be treated as a normal distribution, and even tested for normality. Climate change giving erratic data over time and space for the variables adds to the problem. Generalized linear models (GLMs) are required for this type of problem (McCullagh and Nelder, 1989; Hughes and Madden, 1995; Collett, 2002): In nematode ecology, clustered or aggregated distribution is tested to match the Taylor's Power Law or Neymann's distribution, respectively, to provide the better fit (Dasgupta, 1998, Chapter Ecology, Section 8.4, pp. 276–284; see also: Dasgupta and Rama, 1987; Gaur *et al*., 1988; Mukhopadhyaya and Sarkar, 1990).

A function of the mean, or expected value, of Y is modelled as a linear function of the variables or the factors of interest: the function, called link function can be written as g(μ), where μ is the expected value in the function Y ($\mu = E(Y)$). This approach is different and realistic from the linearization or the regular normal-distribution approach (Garrett *et al*., 2004). Thus, a GLM allows correct calculation of means rather than forcing the researchers to draw inference about the means of such as the arcsine-transformed responses to meet the assumption of normality which is not the biological reality. Fitting GLMs to data necessitates using maximum likelihood, a method based on finding the parameter estimates that result in the highest probability of observing the actual data obtained from discrete distribution such as Poisson and binomial, being appropriate theoretical distributions at least initially for counts and proportions, respectively (Hughes and Madden, 1995; Agresti and Natarajan, 2001; Collett, 2002). Turechek and Madden (2003) suggest some more non-parametric alternatives; more common are the use of curve-fitting with standard equations in mathematical ecology and then to choose the one that fits best. Brunner *et al*. (2002) and Brunner and Puri (2001) suggest more precise approaches, which are likely to attract the interest of the plant-pest scientists more frequently than ever. This method is likely to be more appropriate for selecting disease-resistant varieties or strains or a new technique of plant protection.

The residual error in a model is a random-effects term. Most variables studied by pest scientists created random-effect terms for the models derived (Schabenberger and Pierce, 2002). Garrett *et al*. (2004) commented that all the data analysis claiming LMM models do not truly represent the LMM type. Many computer programs of PROC GLM or SAS (Littell *et al*., 1996; SAS Institute, Cary, North Carolina) actually fit pure fixed-effects models to data, when one uses certain terms as random-effects. In order to produce correct test statistics, standard errors and more, the LMM approach allows for direct incorporation of many types of data and design. Slowly, plant pathologists are adopting MIXED rather than the SAS and its likes they have been using so far. True LMMs do not need sums of squares or mean squares (Littell, 2002), instead they use log-likelihoods or maximum likelihoods. The nematode distribution fits into log normal or negative binomial in different sites. The negative binomial is the most relevant for unbound counts. The k value of negative binomial negatively correlated with the logarithms of the plot area while the b value of Taylor's power law was positively correlated in the case of *Pratylenchus zeae* in a maize field (Gaur *et al*., 1988). Mukhopadhyaya and Sarkar (1990) used the b value of Taylor's power law as an index of aggregation (=2.0)

for *T. zeae*. Lower *b* values indicate low aggregation. *Rotylenchulus reniformis* fitted with both the negative binomial distribution (NBD) and Taylor's power law (TPL) in a brinjal field. Barker and Campbell constructed random and normal distributions of soil texture and population density distribution, *Helicotylenchus digonicus* and *M. arenaria* in a lucerne field with 200 g soil samples from each area of 36 m^2 area in a 7 ha field (Zuckerman and Rohde, 1981; Dasgupta, 1998).

Specialized software and procedures such as SAS and EGRET (Cytel Software Corp, Cambridge, Massachusetts) are more complicated but user-friendly tools for data analysis for different types of spatial disease distribution, disease warning of downy mildew in grapes, and of data from generalized linear mixed models have been developed by different research groups.

4.5.7 Decision theory

This theory provides a framework for evidence-based decision-making in plant pathology, which requires an integrated assessment of the available evidence and associated uncertainty, but there is also emphasis on decision-making for individual plant species or crop field with single or multiple pests with their relative incidence, or other points of their relative healthcare system through successive years in the given crop husbandry history (see for parallelism between human system and crop or cropping system; Gangwar *et al*., 1986b; Dasgupta and Ghosh, 1997; Ashby and Smith, 2000; Mandal *et al*., 2009, 2012). Decision-making also involves establishing linkages and quantifying, rating and integrating them: among the decision-maker considerations are risk factors, possible actions, consequences, utilities; such relates between costs and benefits to the decision-maker such as the farmer or the farming community in the target areas (Swets *et al*., 2000; Mila and Carriquiry, 2004).

4.5.8 Model selection

Use of sensitivity analysis, likelihood and Bayesian methods including risk factor analysis are considered. Still better is the information-theoretic approach as this has been adopted for integrating the information and communication networking through decision transmission system from the modeller to the decision-maker (Mandal, 2012).

4.5.9 Microarray analysis

This kind of analysis is designed for each cDNA sequence analysis in the genetic approach. This may neither fit well nor has any near possibility to be used for epidemiology. For instance, using leaf wetness duration in agro-micrometeorology seems impossible because there is need for continuous recording during each phase of infection and disease development. Incomplete block designs may be used in microarray analysis (Churchill, 2002). Historically, statistical analysis has been used in one field while another identically potential area remains unaware of it. Some mainstream statistical techniques have been and are being developed for each study area. Commercial software(s) is not accessible in poor and developing countries, and not in all institutions.

4.5.10 Pseudoreplication in large-scale ecological studies and in microarray analyses

Hurlbert (1984) defined 'Pseudoreplication as the use of inferential statistics to test for treatment effects with data from experiments where either treatments are not replicated (though samples may be) or replicates are not statistically independent.' Pseudoreplication demands diverse. Such problems arise from ecological survey data being drawn from a large spatial scale of enquiry. If pseudoreplication can be of any relevance, giant meteorological and epidemiological models may be reliable and usable by farmers over more extensive areas. But this has not been discussed by Sanogo and Yang (2004).

4.5.11 Multivariate statistical methods

These are employed to disentangle the nature of a pathosystem such as disease epidemics for descriptive or predictive purposes, measurements of several variables in comparable space and time. For example, it may demand: (i) distinguishing variation among several isolates of the same pathogen based on disease severity; (ii) identification of the most important variables; and (iii) assessment of the potential of developing regional scale versus site-specific post-management schemes using weather and site and site variations. In all these cases, there is a simultaneous handling of several variables such as discriminant analysis, multivariate analysis, multivariate analysis of variance (MANOVA), correspondence analysis and canonical correlation analysis. Dasgupta and Ghosh (1997) used principal component analysis (PCA), discriminant analysis, factor analysis, cluster analysis and path analysis while assessing the potentiality of natural control of pests but not as an overall multivariate analysis, because of ignorance at the time of doing the experiment. Sanogo and Yang (2004) list the multivariate tools and their goals in Table 4.3. Table 4.3 gives a general guide to choosing multivariate statistical tools based on the nature of independent and dependent variables: Table 4.4 gives a general guide to choosing statistical tools based on nature of interdependent variables (see details in James and McColloch, 1990; Hair *et al*., 1998).

4.5.12 MANOVA

MANOVA is a procedure to assess differences among several non-metric independent variables to achieve the simultaneous examination of several dependent variables. In the case of plant-parasitic and plant-pathogenic nematodes and the NPF, the components of the dependent and independent variables are conveniently analysed. Similar association between different plant-pathogenic species in a crop or a cropping system can be conveniently analysed at a number of time points, such as *t1*, *t2*, *t3*, *t4*. MANOVA enables handling of a significant effect; two of the possible

Table 4.3. A general guide to choose the multivariate statistical tools based on the nature of independent and dependent variables (Sanogo and Yang, 2004).

Dependent variable		Independent variable	
Metric only	–	Nonmetric only Multivariate Multiple regression analysis of variance	Metric or nonmetric Multiple regression analysis
Nonmetric only	*Discriminant* analysis	*Correspondence analysis*	Multiple logistic
Metric or nonmetric	Component analysis	–	*Canonical correlation analysis*

Note: Italicized tools are briefly described in the text (details in Hair *et al*., 1998).

Table 4.4. A general guide to choosing multivariate statistical tools based on nature of interdependent variables (Sanogo and Yang, 2004).

Tool	Nature of variables		
1	Metric only	Nonmetric	Metric or nonmetric
Principal component analysis	+	…	…
Principal coordinate analysis	+	…	…
Correspondence analysis	…	+	…
Correspondence analysis	…	+	…
Factor analysis	+	…	…

Details in James and McColloch, 1990; Hair *et al*., 1998.

approaches are: to assess the contribution of the dependent variable, and to make the multivariate analysis. Several criteria are available gauging multivariate differences such as Roy's ger, Wilk's lambda (or, *U* statistic), Hotelling's trace and Pillai's criteria (Stevens, 1992; Hair *et al*., 1998).

PCA enables the identification of linear combinations of dependent variables with the highest variance in the given experiment or the survey. A drawback of using PCA as a remedial measure is that the procedure may lead to difficulties in interpreting the results.

Correspondence analysis describes relationships among two or more cross-tabulated categorical variables (contingency table), frequencies in the contingency table are transformed into chi-square distances to establish a perceptual map of the relations among variables. Savary *et al*. (2000) used correspondence analysis to establish the relationship of 38 plant injury levels and five yield loss levels in rice crop production in tropical Asia, and identified four broad groups of injury corresponding to four loss categories. Correspondence analysis of polymorphic DNA (random amplified polymorphic DNA; RAPD) banding patterns provided a separation of Canadian isolates into 21 groups of *Phytophthora infestans*, Gpi allozyme profile and response to metalaxyl (Mahuku *et al*., 2000). This analysis is widely used in population genetics studies.

Canonical correlation describes the association between two sets of variables. More specifically, Schlosser *et al.* (2000) used rice/*Pyricularia grisea* in the pathosystem of six upland rice cultivars, and used canonical correlation to characterize five plant growth variables and two disease variables. Bullock *et al*. (2002) applied canonical correlation analysis and found significant correlation between the set of variables in biological communities and the set of variables in soil physical and chemical properties.

Redundancy analysis is an alternative to canonical correlation analysis, aiming at measuring the percentage variation in a set of variables (considered singly) that is accounted for by the other set of variables (considered collectively).

Thus, multivariate analysis tools are useful in unravelling patterns in multi-dimensional data from phytopathological studies especially in epidemiology, macro- and micro-agrometeorology, no less importantly in the ecology of plant-parasitic and pathogenic nematodes as well the NF in the present context.

4.6 Conclusions

In so far as nematode management is concerned in the developing countries at this point of time, it is necessary to evolve an appropriate precision agriculture with site-specific cropping system management (APASSCSM) for marginal and small farmers with minimum use of agrochemicals. For the AIPM of plant-parasitic and plant-pathogenic nematodes irrespective of economic and technological capabilities, integrated nematode management has no option, but a compulsion. It has to be evolved as an advisory and appropriate nematode management in site-specific cropping system to be ultimately integrated into the APASSCSM but should particularly cater to the marginal and small farmers in developing countries such as India.

Nematologists all over the world are confronted with the non-availability of effective chemical nematicides coupled with their prohibitive cost and environmental concerns. Therefore, increased research interests are growing on alternative approaches of nematode control with major emphasis on the biological suppression of nematodes through exploitation of native isolates of NF. Biocontrol agents are undoubtedly an effective and ecofriendly means of nematode management. However, limitations include slow acting – not as magical as chemical pesticides – low shelf-life of the formulated products and often non-availability of local isolate-based products. The ideal biocontrol agent should be self-sustaining in the agroecosystem and provide longlasting effects on target pests. The final delivery of biocontrol agents requires mass production and inundation of bioformulations at economical dosage in soil for achieving satisfactory control within a season. A number of biocontrol agents have been investigated against major plant-parasitic nematodes worldwide. However, over the decades, research on biocontrol agents including NF

concluded with a major setback with the inconsistency in experimental and field results. NF displayed great diversity, distribution and wide adaptation to various ecological conditions. A number of NF are known, capable of producing antimicrobial and nematicidal compounds like linoleic acid (*A. oligospora*, *A. conoides*) or pleurotin (*Nematoctonus robustus*, *Nematoctonus concurrens*). However, they are not yet deliverable for management of nematodes. Systematic efforts for the intensive search for the strains of fungal antagonists of nematodes with enhanced efficacy are required. Further, the compatible strains of the fungi could be mixed together (in consortia) and suitability formulated as bionematicide for field application.

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5 Nematophagous Fungi: Virulence Mechanisms

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5.1 Introduction

Plant-parasitic nematodes cause physiological changes and injuries that reduce the absorption and transportation of water and nutrients to the plant, affecting their development, productivity and even product quality. They cause an estimated loss of US$358 billion annually on a worldwide basis (see Abd-Elgawad and Askary, Chapter 1, this volume). The polyphagous habit and the broad distribution of some main species of nematodes hinder the use of more efficient handling methods, the varietal resistance and crop rotation, therefore it is indispensable to use other technologies and tactics to manage nematodes. Biological control stands out as an efficient control alternative: viable and with a lower cost; easy in application; does not cause harm to the environment and human health; does not leave residue in collected products; is an appropriate alternative in short-cycle crops; does not allow the emergence of resistant forms of nematodes; does not cause imbalance of the soil biota; may transform a conducive soil from suppressive soils; and collaborates to the integrated handling of nematode management in sustainable agriculture (Soares, 2006).

Biological control aims to reduce the nematode population or their capacity to feed on or cause damage to plants through the action of one or more living organism that occur naturally in the soil, or through the manipulation of the environment including the introduction of antagonist organisms (Baker and Cook, 1974). It is based on the antagonist relationship between microorganisms and phytopathogenic, such as nematodes, being characterized by different types of action: antibiosis, predation, plant host resistance induction, microparasitism, enzyme and toxin production, systemic colonization of the plant host, competition of nutrients at colonization sites and liberation of hydrolytic enzymes that act in the degradation of the cellular wall (Bettiol, 1991; Melo and Azevedo, 1998).

Among these organisms, around 75% of antagonists already identified as possible agents in the biological control of nematodes

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and presenting a highlighted position are the fungi that are normally found in soil and are harmless to the crops and human health, but parasitize eggs, prey on juveniles, adults or cysts, or even produce substances toxic to nematodes (Van Gundy, 1985; Jatala, 1986; Santos, 1991; Stirling, 1991).

The antagonist fungi of nematodes can be divided into two main groups: those using nematodes as a nutrient source and others that cause an adverse effect on the nematodes without using them as a nutrient source. Some nematophagous fungi are obligate parasites, others are facultative parasites and others have intermediate characteristics between the two groups. The nematophagous fungi can also be divided into those that form intense growth of hyphae, such as predators and nematode egg parasites, and those that are predominantly endophytic (Viane *et al*., 2006).

5.2 Virulence Mechanisms of Predaceous Fungi

The predatory nematophagous fungi can be defined as those that capture the nematode, penetrate their cuticle cells through the hyphae with the objective of colonizing and consuming their body contents, ultimately leading to the death of the nematode. These antagonists constitute one of the most studied groups and are promising biological control agents of nematodes. The quick growth and intense mycelial development are the key attributes for their dissemination and survival in the natural environment (Dackman *et al*., 1987).

Predatory fungi produce specialized structures for capturing the nematodes through their hyphae, generally referred to as traps. These structures can be adhesive or not (Nordbring-Hertz, 1972). Seven types of traps are known: (i) three-dimensional adhesive nets; (ii) two-dimensional adhesive nets; (iii) adhesive nodules; (iv) adhesive branches; (v) constrictor rings formed by three cells; (vi) non-constrictor rings; and (vii) non-modified adhesive hyphae that adhere to the nematode's body. These structures are shown in Fig. 5.1. There are fungi that form specialized structures to capture nematodes, but the hyphae penetration by the fungi occurs directly on the nematode's cuticle, involving mechanical action (pressure) (Soares, 2006). Some trap variants are shown

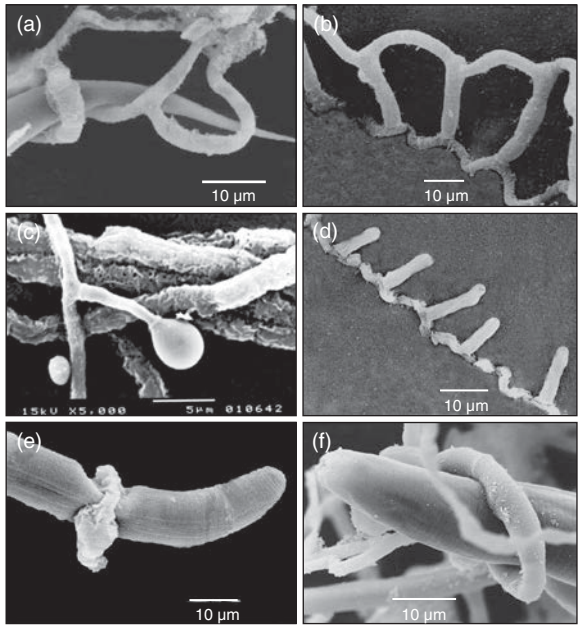

Fig. 5.1. Some specialized trapping devices produced by predaceous fungi: (a) three-dimensional net; (b) two-dimensional net; (c) adhesive nodule; (d) adhesive branches; (e) constricting ring; and (f) non-constricting ring.

in Fig. 5.2. Some trap-forming nematophagous fungi are: *Arthrobotrys dactyloides*, producing constrictor rings; *Dactylella candida*, producing non-constrictor rings and adhesive nodules; *Monacrosporium cionopagum*, producing adhesive branches and three-dimensional adhesive nets; and *Monacrosporium ellipsosporum*, producing adhesive nodules and adhesive nets (Viane *et al*., 2006). Usually, the adhesive nodules and rings are found in alternation in the same predator fungus hyphae (Gray, 1988).

There are more than 50 species of predatory nematophagous fungi that are capable of capturing and killing nematodes in the soil (Dijksterhuis *et al*., 1994; Siddiqui and Mahmood, 1996). The most studied predatory fungi belong to the genus *Arthrobotrys*, *Dactylaria*, *Dactylella* and *Monacrosporium* (Mankau, 1980). The species of *Arthrobotrys* and *Monacrosporium* are some of the most common predaceous fungi in Brazilian soil (Soares, 2006). The genus *Arthrobotrys* comprises a number of species that form one of the most important groups of nematophagous fungi. They are intensely studied with regards to their ecology and pathophysiology, and are frequently included in nematode handling research (Dijksterhuis *et al*., 1994; Siddiqui and Mahmood, 1996; Soares, 2006; Cardoso *et al*., 2009).

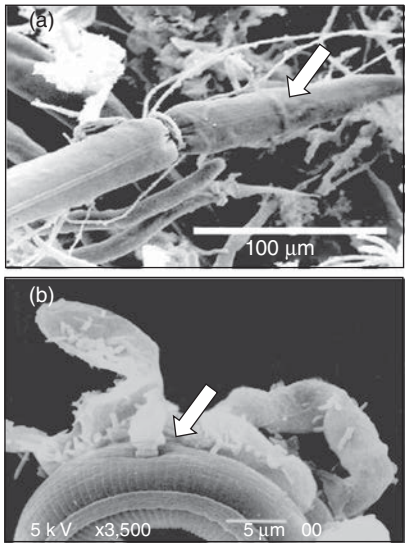

Fig. 5.2. Nematodes trapped by fungal trapping devices: (a) a typical constrictor ring not formed by three dilated cells, developing hyphae in the interior of the nematode's body (arrow); and (b) direct penetration of fungi on the nematode cuticle (arrow) showing mechanical action.

Each of the traps formed by nematophagous fungi has intrinsic characteristics that can determine their efficiency as biological control agents of phytonematodes. Usually the same nematophagous fungus may present more than one type of trap, as for example *Dactylella leptospora* forms a non-constrictor ring and adhesive nodule, *Arthrobotrys oligospora* forms tri-dimensional nets (Silveira *et al*., 2001).

5.2.1 Adhesive hyphae

Adhesive hyphae that deposit certain materials at some points to trap the nematode in contact are frequently found in fungi belong to Zygomycetes. The absence of a septum in these hyphae does not allow the formation of complex structures for the capture of nematodes (Barron, 1977). These fungi produce adhesive material that is deposited at some points on the hyphal surfaces. When the nematode touches these points, it becomes captured. The hyphae produce appressoria that penetrate through the nematode wall, and the mycelium grows throughout the nematode body; after consuming all the nematode contents, the fungus withdraws total plasma to outside the nematode body for the development of sporangia and spores (Chen and Dickson, 2004).

5.2.2 Adhesive branches

Adhesive branches behave similarly to the adhesive hyphae that form entangled adhesives or two-dimensional nets like hose in *Monacrosporium robustum* (Maia *et al*., 2001). They appear to be very effective in the capture of nematodes; once caught by hyphae, they increase the intensity of their movements in an attempt to escape and end up being caught in several hyphae. This type of structure is efficient in capturing larger as well as smaller nematodes (Chen and Dickson, 2004).

5.2.3 Adhesive nets

This type of structure is usually found in the fungi belonging to Deuteromycetes (Chen and

Dickson, 2004). The adhesive nets may form two-dimensional or three-dimensional nets like in *A. oligospora* (Silveira *et al*., 2001; Martinelli and Santos, 2010). Martinelli and Santos (2010) demonstrated with the help of electronic scanning microscopy (ESM) the formation of these three-dimensional nets by *Monacrosporium elegans* and two-dimensional nets by *Monacrosporium eudermatum*.

5.2.4 Adhesive nodules

Adhesive nodules formed by fungi are highly specialized cells that produce adhesive substances; they can be sessile or can occur at the top of a non-adhesive stem. When the nematode contacts the connection point of the nodule it is stimulated immediately, producing an adhesive substance that firmly sticks to the nematode. Generally the nodules are well spaced throughout the hyphae, and in the struggle to escape the nematode becomes adhered to many of these nodules (Gray, 1988). The nodules end up being released with the movement of the nematode, which is paralysed after the penetration of its body. This type of capture structure is produced by *D. leptospora* (Silveira *et al*., 2001) and *D. candida* (Barron, 1977).

5.2.5 Non-constrictor rings

Non-constrictor rings are formed in branches that emerge from septate hyphae of the fungi belonging to Deuteromycetes, e.g. *Dactylaria.* This type of structure only captures nematodes with a bigger diameter than the ring. This ring-type structure is formed in a delicate stem, which a nematode can easily break and carry many such rings until it is immobilized and parasitized (Chen and Dickson, 2004).

5.2.6 Constrictor rings

The constrictor ring is the most sophisticated type of trap, frequently found in *Dactylella*, *Dactylaria* and *Arthrobotrys*, and are different from the non-constrictor rings as they are formed on a difficult to break strong stem. The diameter of the ring varies according to the species of fungi; if a nematode moves inward, the three ringed cells are stimulated and rapidly triple their volume, which results in the reduction of the ring's opening and consequently the nematode is captured (Keke *et al*., 2012) and does not have a chance to escape or, if that occurs, it will die, since part or the entire trap is adhered to the nematode and has parasitized it. Probably the expansion of these cells is due to a rearrangement of water molecules and the existing colloidal substance in the cell, caused by mechanical stimuli (Chen and Dickson, 2004). The colloidal substance seems to be related to the permeability change of the cell membrane, which is due to higher absorption of water leading to closing of the ring (Muller, 1958). This type of structure was recently reported in Brazil in *D. leptospora* parasitizing nematodes of citrus, *Pratylenchus jaehni* (Martinelli and Santos, 2010).

5.2.7 Process of nematode infection

Some fungi produce traps spontaneously whereas some produce these structures in response to environment stimuli. There is evidence that the formation of adhesive nodules is autonomous and independent from the presence of nematodes and exogenous nutrients (Boogert *et al*., 1992). Li *et al*. (2011) observed the increase in the formation of traps of *A. oligospora* when cultivated in conjunction with isolates of *Chryseobacterium* sp. Other researchers are of the opinion that the formation of fungi traps is influenced by the addition of nutrients. The lowest level of nutrition was observed when there was higher formation of fungal traps. Furthermore, the pH and soil humidity are the factors that contribute the most to the presence of predatory fungi, whereas the endoparasitic fungi are influenced by the organic matter present in the soil (Gray, 1985).

The infection process involves a chain of events such as attraction, apprehension and penetration. Several factors are involved in attracting nematodes, such as carbon dioxide (Robinson, 1995) and organic and inorganic substances. Silicic acid has a fundamental role

in the attraction of nematodes, such as adhesion of conidia to the cuticle of the nematode in the case of endoparasitic fungi (Jansson and Nordbring-Hertz, 1984). The intensity of attraction of nematodes by nematophagous fungi is directly related to the increase in the dependence for nutrients (Jansson and Nordbring-Hertz, 1979).

The apprehension of nematodes is a result of morphologic modifications and biochemical interactions between the nematode cuticle and the fungus. The adhesive traps produced are accumulated in the apprehension place (Ahrén and Tunlid, 2003). In some nematophagous fungi, these are associated with the formation of adhesive cells. During the process of nematode capture, extra-cellular polymers, adhesive substances that contain lecithin such as N-acetylgalactosamine, are involved in the process of recognition of the nematode and adhering to specific carbohydrates of its cuticle (Kaplan *et al*., 1991).

When the nematode is captured in fungal traps the hyphae penetrate the nematode cuticle and, afterwards, form an infection bulb. The penetration of cuticle is the combined result of weakening caused by the enzymatic action and application of mechanical force (Dijksterhuis *et al*., 1990). These events are followed by the fungal colonization of the nematode body and, afterwards, the formation of conidiophores containing conidia takes place that emerge from the nematode cadaver. In the capture process and colonization of nematodes, the nematoxins produced by the traps can help in its immobilization. The three-dimensional nets formed by *A. oligospora* produce a protease PII (serine) that potentially has a role in the immobilization of nematodes; whereas, some isolated with super-expression from gene PII of the protease demonstrate an increase in virulence (Morton *et al*., 2004).

5.3 Virulence Mechanisms of Endoparasitic Fungi

Endoparasitic fungi are different from the predaceous fungi as the former have no specialized trapping structure to capture vermiform nematodes. In the beginning very little work was performed related to their biocontrol efficacy, mainly due to the fact that the endoparasitic fungi of nematodes are normally obligate parasites with low saprophytic ability (Barron, 1987). Few endoparasitic fungi species are readily cultivated *in vitro* or complex methods are required, which hinders the development and use of these fungi. On the other hand, useful endoparasitic fungi frequently produce resistance structures to assure their survival in the soil when the hosts are limited (Santos and Ferraz, 2000).

Endoparasitic fungi may be categorized in four groups according to their mode of infection: (i) cyst fungi; (ii) fungi forming adhesive conidia; (iii) fungi forming conidia that can be ingested by nematodes; and (iv) fungi that form gun cells (Barron, 1987; Chen and Dickson, 2004). After infecting the nematode, the fungal structures consume the host body and produce conidia (Barron, 1977).

5.3.1 Encysting fungi

The cyst fungi produce zoospores that have the ability to encyst the cuticle of vermiform nematodes. The most studied fungus in this group is *Catenaria.* The spores of *Catenaria anguillulae* have been found to have high virulence capacity against the young wheat seed gall nematode, *Anguina tritici*, which makes the fungus a promising biocontrol agent (Singh *et al*., 2012). *C. anguillulae* is part of the Chytridiomycota group, the only major group of true (chitin-walled) fungi that produce motile spores, termed as zoospores. This fungus can be grown easily on culture media, but in nature it is often found as a facultative (non-specialized) parasite of nematodes, the eggs of liver flukes or other small organisms (Deacon and Saxena, 1997).

5.3.2 Conidia-producing fungi

Some species of nematophagous fungi form adhesive conidia that after maturity are liberated into the soil, and when they come into contact with a nematode, start germination and infection. This type of structure is found

in *Nematoctonus*, *Verticillium*, *Cephaloporium*, *Acrostolagmus* and *Hirsutella* species (Chen and Dickson, 2004).

5.3.3 Ingestible conidia-producing fungi

Some endoparasitic fungi form special conidia and have great morphological variability. These conidia are ingested by nematodes and eventually become established in the oral cavity and oesophagus. *Harposporium* species are mostly found in soils, and are capable of producing such a type of conidia (Drechsler, 1963; Chaverri *et al.*, 2005). The first such report was made by Lohde (1874) in the case of *Harposporium anguillulae*. This endoparasite produces sickle-shaped conidia on slender sterigmata arising from globose conidiogenous cells that are borne on the lateral parts of the filamentous hyphae extended from the nematode body. Only 20 species of *Harposporium* reported to have been cultured artificially are capable of producing more than one type of conidia (Wang *et al.*, 2007).

5.3.4 Fungi that produce gun cells

Haptoglossa species produce zoospores that encyst when in contact with substrate; after some hours they form special cells called 'gun cells', that have a cannon or revolver aspect. The *Haptoglossa* gun cell is the most complicated cell known in the fungi and arguably more sophisticated than the 'nematocyst'. It shoots off an infective sporidium into a moving rotifer or nematode in a fraction of a second. These cells upon making a contact with nematode, cause discharge of an infective sporidium that penetrates the cuticle; the subsequent events lead to colonization of the nematode's interior (Barron, 1987).

5.4 Virulence Mechanisms of Toxin-producing Fungi

The fungi that produce toxic metabolites represented by the species of *Aspergillus*, *Pleurotus*, *Penicillium*, *Trichoderma* and *Myrothecium* demand more study under the effect of possible toxic substances produced by such fungi in the management of phytonematodes (Ferreira *et al.*, 2008). Several fungi are capable of producing metabolites toxic to vermiform nematodes that immobilize females and finally check the outbreak of nematodes. These metabolites can act in more than one state of the nematode or have a specific action to certain states as acknowledged by Costa *et al*. (2001). The filtrates of *Paecilomyces lilacinus*, *Fusarium moniliforme* and *Fusarium oxysporum* caused mortality of root-knot nematode, *Meloidogyne incognita*, while the other fungi such as *Fusarium solani*, *Aspergillus flavus*, *Cylindrocarpon magnusianum* and *Mortierella* sp. limited the nematode population.

Few studies have been performed in order to characterize these substances and the metabolic events that cause the death of nematodes. *Trichoderma harzianum* has been found capable of penetrating the matrix of the egg mass and reducing the nematode population, probably by the increase in chitinase extra-cellular production, apart from stimulating the peroxidase enzymes (POX), phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO), related to the resistance induction of plants (Sahebani and Hadavi, 2008).

5.5 Virulence Mechanisms of Egg- and Female-parasitic Fungi

The egg-parasitic fungi or opportunistic fungi are the most promising, due to their saprophytic ability and the ease of growing *in vitro*. Normally, these fungi are saprophytic; they do not depend on the presence of nematode eggs in soil for their existence and grow satisfactorily in organic material. Due to these characteristics, they are easier to establish in the soil. They colonize eggs and females rapidly, destroying at once a large quantity of individuals, especially in the case of gall and cyst nematodes (Stirling, 1991). *P. lilacinus* releases chitinase and protease capable of causing deformation in the nematode eggs, with the appearance of big vacuoles in the lipid layer, drastic reduction of the chitin layer and

the division and loss of the vitelline membrane (Khan *et al*., 2004). *Meloidogyne* spp. can be controlled very efficiently by this group of fungi because the egg mass of these nematodes is compact in a gelatinous matrix which is colonized by the fungus (Abad *et al*., 2009) and as a consequence reproductive capacity of the nematode decreases sharply (Barron, 1977; Mizobutsi *et al*., 2000). Furthermore, the egg membranes of the nematode, which are composed mainly of chitin, are damaged as these fungi have chitinolytic activities (Jatala, 1986).

Pochonia chlamydosporia and *P. lilacinus* have been the most studied fungi of this group. *P. chlamydosporia* is an egg parasite of root-knot nematodes (Monfort *et al*., 2005). This fungus is also referred to as a gall nematode control agent, since it prevents egg formation (Morgan-Jones *et al*., 1983). The fungus action may occur both on the eggs and on the young. It has been observed that the egg cuticles of *M. incognita* were broken and the fungal hyphae were profusely developed. The parasitic activity is due to the enzymatic breakdown and the physiological disturbances generated by the toxic metabolites biosynthesis (Morgan-Jones and Rodriguez-Kabana, 1985). *P. chlamydosporia* also releases some proteases that hydrolyse the external layers of the egg wall, which results in exposing the chitin layer. *P. chlamydosporia* and *Pochonia lecanii* isolates release protease that is pathogenic to nematodes, while other species of *Pochonia* that parasitize plants do not release these enzymes (Segers *et al*., 1994). In the absence of nematodes they can develop saprophytically (Siddiqui and Mahmood, 1996). The production of chlamydospores, i.e. resting structures, is another characteristic of this fungus that offers advantage in terms of long durability as well as formulation of products (Kerry, 2001). It should be noted that differences exist within the isolates that can influence the capacity of parasitism. Morton *et al*. (2003) reported a genetic difference in the production of protease, VCP1 obtained from the isolates of *P. chlamydosporia*, isolated from the nematodes of different geographic regions, indicating that this can be related to the specific host preference.

Early in 1978, *P. lilacinus* was isolated from the egg masses of *Meloidogyne* species, attacking potato roots in a central region in Peru (Jatala, 1986). The egg-parasitic fungus with limited host specificity was placed in the class Moniliales (order: Hyphomycetes) (Goettel *et al*., 2001). The fungus has the ability to penetrate nematode eggs and destroy the embryo (Novaretti *et al*., 1986). The fungus consumes all the egg contents. When making contact with the egg, the hyphae fixate on the external walls and produce chitinase, breaking the chitinase-protein complex, allowing the penetration and colonization of all the internal content (Figs 5.3a,b). The fungus grows rapidly and parasitizes all the eggs. Sometimes the predator fungi also colonize the nematode eggs externally. The hyphae wrap the egg and apply pressure over them (Fig. 5.3c). Since these fungi do not produce chitinase, there is no penetration. The cyst and gall nematodes are more vulnerable to their actions, as they deposit the eggs in agglomerates, in the cysts or external masses of the roots, enabling the colonization. In studies conducted by Holland *et al*. (1999), *P. lilacinus* 251 infected *Meloidogyne javanica* eggs of all stages, including those containing unhatched juveniles. The juveniles pertaining to the third (J_3) and fourth stages (J_4) together with the *M. javanica* adult females were infected by hyphae that penetrated through their body wall and formed conidiophores. *P. lilacinus* can grow in a great variety of substrates, adapt to a large pH range of soil and is very competitive in the field (Jacobs *et al*., 2003). The effect of fungus on the eggs of *M. javanica* showed that the disintegration of the vitelline, chitin and lipid layer of eggs was caused solely by the enzymatic degradation of protease and chitinase. Furthermore, the enzymes can contribute in the physical penetration of the fungus and improve the efficiency of infection (Carneiro and Gomes, 1993; Khan *et al*., 2004). The synergy of protease and chitinase of *P. lilacinus* were capable of reducing significantly the development and production of *M. javanica* eggs (Huang *et al*., 2004). A serine protease of *P. lilacinus* was responsible for the lesion caused to the shell of *Meloidogyne hapla* eggs, playing a key role in the fungus pathogenicity (Bonants *et al*., 1995). In addition to the serine protease, chitinase activity was also observed in supernatants of *P. lilacinus* culture (Khan *et al*., 2004). The action that combines the protease and the chitinase

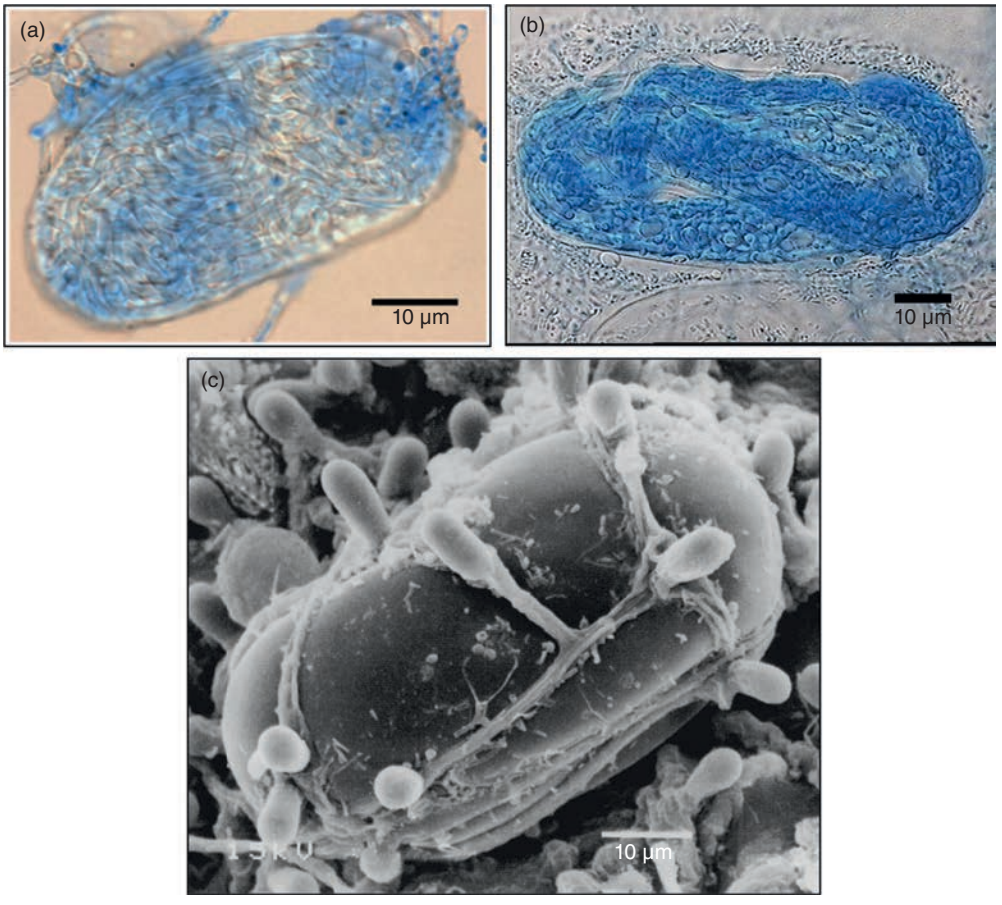

Fig. 5.3. Nematode eggs colonized by fungi: (a) an egg in embryogenesis development stage colonized by *Paecilomyces lilacinus*; (b) an egg containing a juvenile colonized by *P. lilacinus*; and (c) an egg externally colonized by a predator fungus, *Monacrosporium robustum*.

destroys the lipid layers of egg, induces hydrolysis of chitin and alters the vitelline layer (Tikhonov *et al*., 2002).

The opportunistic fungus *P. lilacinus* was internally and externally isolated from females of *Meloidogyne* spp. and analysed *in vitro* against the females of *M. javanica.* All isolates demonstrated high parasitism ability, indicating that the female's body constitutes a good nutritional source for these fungi (Coimbra *et al*., 1999).

Trichoderma species are considered efficient antagonists against a series of fungi, acting in the production of volatile and non-volatile metabolites (Claydon *et al*., 1987). Species of this fungus have been used in biological control of several phytopathogenic fungi (Martins-Corder and Melo, 1998; Ethur *et al*., 2005; Patrício *et al*., 2007) as well as root-knot nematode, *Meloidogyne* spp. (Sharon *et al*., 2007; Ferreira *et al*., 2008). The potential of *Trichoderma* spp. in the management of nematodes is due to the production of inhibitory volatile metabolites and production of lytic enzymes that degrade the chitin of nematode eggs (Santin, 2008). The enzymatic activity was reported when the culture filtrates of *P. lilacinus* and *Trichoderma* spp. were used against nematodes (Sharon *et al*., 2007; Santin, 2008; Freitas *et al*., 2012). The chitinolytic activity of these fungi is probably most effective in causing the egg's sheath lesion (Romao-Dumaresq *et al*., 2012). In tests performed

in vitro there was no colony growth of *Trichoderma* sp. upon *P. chlamydosporia* in the direct confrontation tests. In the antibiosis test, there was no antibiotic production in the centre of the culture, which resulted in the absence of an inhibition halo. The combined application of these two fungi may favour nematode control as well as other fungi that cause plant disease. It was concluded that the isolates were efficient in colonizing eggs of *Meloidogyne exigua* (Ferreira *et al*., 2008). The infection mechanisms used by these ovicidal fungi can be mechanical, enzymatic or both (Bonants *et al*., 1995). The identification of numerous extracellular enzymes has been confirmed as an important factor of virulence associated with the infection process (Huang *et al*., 2004). Although the pathogenicity of egg-parasitic fungi is not completely clarified, evidence shows that extracellular hydrolytic enzymes, including protease, collagenase and chitinase, may be involved in the nematode cuticle penetration and digestion (Morton *et al*., 2004; Yang *et al*., 2007).

5.6 Conclusions

In an agroecosystem there are numerous nematophagous fungi with different action mechanisms and a good potential to manage phytonematodes. However, in general, predatory and egg-parasitic fungi are the most studied, due to their ability to adapt to the agricultural environment, their ease of production in the laboratory and of course their nematode control potential. It is important to note that under natural biological control or applied control, these fungal biocontrol agents with different action mechanisms can play an important role to bring the nematode populations below a threshold level. This is due to the fact that these fungi generally have a determined specificity against certain stages or species of nematode pests. Therefore, it can be concluded that by identifying and considering the specificity of these fungal biocontrol agents against the developmental stages of certain nematode species, the management of phytonematodes can be done successfully.

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6 Nematophagous Fungi: Formulation, Mass Production and Application Technology

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6.1 Introduction

A successful plant-parasitic nematode (PPN) management requires a combination of management tactics, such as exclusion measures, crop rotation, use of antagonistic plants, resistant varieties and chemical and biological methods. Among these, the biological method of nematode management by using nematophagous fungi has drawn considerable attention by researchers all over the world (Barron, 1977; Fattah, 1988; Maia *et al*., 2001; Bernardo, 2002; Corbani, 2002; Martinelli *et al*., 2012a,b). These carnivorous fungi are the most studied organisms for the management of nematodes. The first report of fungi parasitizing nematodes was reported by Zopf (1888), and the first attempt of using these microorganisms in nematode control was taken by Cobb in 1920 (Freitas and Ferraz, 2005). From the early work to the present day, several studies have been conducted with nematophagous fungi, however, biological control is still one of the least studied alternatives, since only a few researchers have devoted their work to this study area. The research has focused on the potential agents of biological control, in spite of the favourable evidence in practice towards the success of this alternative; the studies performed under field conditions are also not numerous (Stirling, 1991; Martinelli, 2008; Crow, 2013; Manzanilla-Lopez *et al*., 2013).

The biological control of nematodes involves either: (i) reduction of the nematode population by the action of other living organisms that are naturally present in the soil; or (ii) manipulation of the environment by the artificial introduction of antagonistic organisms. This technique presents enormous potential for application in protected cultivation such as vegetable production, irrigated fruit farming and organic agriculture as well as in urban parks and gardens (Soares, 2006; Martinelli, 2011). According to Soares (2006), biological control of nematodes has many advantages over the chemical control.

1. Easy in application.
2. Does not imply harmful effects to the environment.
3. Does not leave residues in harvested products.

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4. Does not favour the emergence of nematode resistant forms.
5. Does not affect the soil biota, thus preventing the resurgence of a more severe problem.
6. May transform potentially conducive soil into a suppressive one.

Therefore, the ultimate aim is to exploit the nematophagous fungi against nematodes under pot and field conditions to protect the plant from nematode attack and increase the crop yield. This necessitates research on mass production, proper formulation and application technology so that maximum benefits may be achieved from these biocontrol agents (BCAs).

In the present article, we have made an attempt to highlight the ideas involved in mass production, formulation and application of nematophagous fungi under field conditions and also throw light upon the limitations involved in their commercialization.

6.2 Nematophagous Fungi: Formulation

In recent years, several researchers have tried to formulate nematophagous fungi for use for commercial purposes and in the process a little success has been achieved (Prabhu *et al.*, 2008; Crow, 2013). According to Freitas and Ferraz (2005), the following are the prerequisites for the formulation of a BCA.

1. Survival ability in the absence of host.
2. Non-host specific and ability to parasitize a variety of nematode species.
3. Efficacy in reducing high densities of PPN populations.
4. Easy and economic in mass production.
5. Greater shelf life.
6. Non-toxic to plants, humans and other animals.
7. Ability to colonize the soil soon after its application.
8. Compatible with fertilizers and other agrochemicals.

Several workers have successfully prepared cereal seed substrate formulations of nematophagous fungi (Bernardo, 2002; Martinelli *et al.*, 2009, 2012a). Among the substrates tested, the antagonistic fungi *Arthrobotrys conoides*, *Paecilomyces lilacinus* and *Pochonia chlamydosporia* were the most promising in rice bran (Machado and Campos, 1997). As discussed above, one of the key points in effective formulation of nematophagous fungi is its viability and stability. Carneiro and Gomes (1997) prepared a pellet formulation of *P. lilacinus* using a mixture of sodium alginate, clay and streptomycin sulfate. They observed that fungus feasibility in the formulation remained unchanged for 5 months at room temperature and for 12 months at 7°C but rapidly decreased after these periods. Soares (2006) researched a viable alternative for the use of by-products in nematophagous fungi formulations. He used broken rice, rice bran, rice husk, guava seeds, beer fermentation by-products, sugarcane bagasse and mixtures of sugarcane bagasse with rice bran and guava seeds with rice bran, all blended with water. These substrates were sterilized by autoclaving at 120°C for 40 min in polypropylene bags. It was found that nematophagous fungi showed better growth on rice bran substrates. However, sugarcane bagasse + rice bran mixture emerged as the best combination for the formulation of nematophagous fungi due to raw material abundance, good aeration and surface characteristics for the growth of fungal hyphae. A formulation containing sugarcane bagasse, rice bran and water was used to formulate a cocktail (mixture of five fungal species, *Arthrobotrys robusta*, *Arthrobotrys oligospora*, *Arthrobotrys musiformis*, *Dactylella leptospora* and *Monacrosporium eudermatum*) of nematophagous fungi for the biological control of lesion nematode, *Pratylenchus jaehni* and citrus nematode, *Tylenchulus semipenetrans*. An excellent result was obtained when the formulation was applied under field conditions, the nematode population decreasing by approximately 90% in citrus orchards (Martinelli, 2008, 2011; Martinelli *et al*., 2012a,b).

There are some commercial formulations of predatory fungi in the world market. However, such formulations have shown variable performances in relation to quality control and, thus, have been little used. In France, formulations like Royal 300® and Royal 350® have already been marketed (Cayrol *et al*., 1978; Cayrol and Frankowski, 1979). Royal 300® was based on the fungus *A. robusta* and used for the control of *Ditylenchus myceliophagous* in mushrooms.

Royal 350® was based on *Arthrobotrys irregularis* for the control of *Meloidogyne* spp. in tomato plants. These biological nematicides, which are not produced anymore, were replaced by other products such as Nematus®. A product named Nemout® based on a mixture of predatory fungi in wettable powder formulation was produced by an American company and was found efficient in reducing galls and eggs of *Meloidogyne javanica* on tomato roots under greenhouse conditions (Al-Hazmi *et al*., 1993). In Russia, two lineages of *Arthrobotrys* are available as 'Nematofagin-BL®', a bionematicide (Matskievich, 1993). Both lineages are effective in reducing the nematode population in different crops. Stirling and Mani (1995) produced granular formulations of *Dactylella candida* and *Arthrobotrys dactyloides* in pure alginate, encapsulating different amounts of fungal biomass. The best formulations were those that were encapsulated in alginate granules. They produced a trapping net in the soil that was maintained for approximately 10 days, corresponding to the bulb volume of 5–10 mm diameter, which was equally distributed around the granule. In 1998, Abbott Laboratories Company launched a biological nematicide in liquid medium derived from the fermentation of *Myrothecium verrucaria* (Ditmar isolate). The product was commercially named DiTera® and was launched in the USA and several other countries for the management of different economically important nematode species in the field, which included root-knot nematode (*Meloidogyne* spp.), cyst nematode (*Heterodera* and *Globodera* spp.), root-lesion nematode (*Pratylenchus* spp.), burrowing nematode (*Radopholus similis*), sting nematode (*Belonolaimus* spp.), stubby root nematode (*Trichodorus* spp.) and many other plant-parasitic species of nematodes associated with different crops (Warrior *et al*., 1999). According to Kerry (1990), since 1981 in the Philippines, the studies on the biological control of nematodes were mainly concentrated on the application of *P. lilacinus*. Some of these accounts indicate that *Meloidogyne* sp. in tomato, *Globodera rostochiensis* in potato, *R. similis* in banana, *T. semipenetrans* in citrus, *Rotylenchulus reniformis* in pineapple and *Pratylenchus* spp. in different crops were found efficiently controlled by a commercial product of this fungus, known as Bioact®, produced in that country. A single fungal isolate (Phil. Strain No. 1) that presented great effectiveness in the colonization of nematode eggs has been mass produced and commercialized by Asiatic Technologies Incorporation, Manila, under the trademark BIOCON® (Davide *et al*., 1990). Paecil®, a biological nematicide prepared from an isolate of *P. lilacinus*, has been commercialized and patented by Australian Technological Innovation Corporation Private Limited (Kerry, 1989). In Cuba, a commercial product based on *P. chlamydosporia* var. *catenulata* was launched and registered under the trademark KlamiC®. The use of this product resulted in more than 80% reduction in nematode infestation (Hernández and Hidalgo Díaz, 2008). Currently, in Brazil there are several products for nematode control based on nematophagous fungi; however, not all of them have proved promising. In 2009, a biofactory was established in Brazil with the aim to produce a biological nematicide based on the fungus *P. chlamydosporia* var. *chlamydosporia*. As a result of this investment, a product Rizotec® was launched. This product has an ovicidal effect and was found efficient in controlling root-knot nematodes. According to the company, the Rizotec® sales have been increasing around 30% per year, resulting in revenues of US$500,000 (Rizoflora, 2013). Field studies on nematophagous fungi formulations developed by Soares (2006) and Martinelli (2008) resulted in a biofactory installation at the State University of São Paulo, Brazil. This experimental biofactory works to produce formulations of nematophagous fungi based on sugarcane bagasse and also attain an increasing demand for viable alternatives required for nematode management. Another objective of this biofactory is to replace the lack of registered products meant for pathogen management based on the need of marginal growers of fruits and vegetables (Silva *et al*., 2009; Aminuzzaman *et al*., 2013; Jamshidnejad *et al*., 2013).

6.3 Nematophagous Fungi: Mass Production

In the mass production process of nematophagous fungi, there is a demand to use pure culture in the substrate inoculation so that it may result in a better formulation. During formulation, culture media should be of such

a type that would promote fast fungal growth and/or sporulation. These culture media must possess the character of simple composition and easy preparation favoured by profuse production of mycelia and spores as these characters are essential for dissemination and survival of the fungus under natural conditions that ultimately are the results of an effective use of BCA (Jansson, 1982; Duan *et al*., 2008; Maareg *et al*., 2008; Prabhu *et al*., 2008). Above all, it should be cost effective so that it may be utilized on a large scale by the growers or farming community.

There are few options of solid culture media that are used for the multiplication of fungi on Petri dishes. The most frequently used media are PDA (potato dextrose agar); PDA + peptone; CMA (corn meal agar), corn meal + agar and YpSs (yeast extract) (Dias and Ferraz, 1993), PDA and YpSs (Machado and Campos, 1997) and PSA (potato sucrose agar) (Castro *et al*., 2000). Some of these are industrialized and few products used in the composition are synthetic and, therefore, the use of synthetic or industrialized products may not be appropriate for studies on the potentiality due to technical difficulties found in the media cultivation of some organisms (Van Gundy, 1985). Studies confirm the favourable temperature for the growth of most of the nematophagous fungi to be 25°C (Tedford *et al*., 1995; Velvis and Kamp, 1996; Castro *et al*., 2000). Cooke (1963) obtained higher mycelial growth from an *A. musiformis* isolate maintained at 25°C. Dias and Ferraz (1993) observed that for the isolates of five *Arthrobotrys* species, the YpSs medium (yeast extract and starch) at a temperature of 25°C promoted higher mycelial growth and sporulation. Although the isolates were from different species and sometimes even from different regions, they showed a similar behaviour with respect to temperature. Castro *et al*. (2000) also reported that the optimum temperature for the production of greater mycelium mass from an *A. musiformis* isolate was 25°C.

6.3.1 Capsule formulation in sodium alginate

Capsule formulation in sodium alginate is a technique used to encapsulate conidia of the isolates of nematophagous fungi. It consists of *in vitro* multiplication of fungal isolates in either solid and/or liquid medium for the production of fungal conidia. The produced conidia are suspended in sterile water and quantified. For capsule formulation, a mixture of sodium alginate (1%), clay (5% sodium bentonite) and streptomycin sulfate (1%) is used according to the methodology described by Carneiro and Gomes (1997). The conidial suspension of nematophagous fungi is added to the mixture and homogenized in a blender. The formulation concentration depends on the amount of added conidia. After the concentration adjustment, $CaCl_2$ solution of 0.25 M is added for the formation of solid aggregates (pellets), which are submitted to a drying process in a laminar flow chamber for 6 h. This process will yield approximately 3.4 g pellets for each 100 ml of suspension (Carneiro and Gomes, 1997). The formulation based on sodium alginate may be stored at ambient temperature, protected from light, for 12 months. The viability of the conidia depends on the formulation of fungus species, however, there may be some inconsistency in the reports on viability. A few workers have reported a viability period of 12 months (Carneiro and Gomes, 1997) whereas others mention viability losses within that period (Cabanillas *et al*., 1989).

6.3.2 Formulation in rice and sorghum grain

Shelled rice (*Oryza sativa* L.) grains are a good substrate for the multiplication of nematophagous fungi. However, for the formulation of nematophagous fungi, the substrate should not be used as a basic human food, which is a great inconvenience with rice utilization. This methodology is very simple and easily practicable. Rice grains are simply washed two to three times under tap water and placed in a container, autoclaved at 120°C for 20 min at 1 ATM (atmospheric) pressure. Before autoclaving, rice grains must be soaked in water in a volume ratio of 2:1 (water:rice) for 10 min. The excess water is decanted (Freitas *et al*., 1999). This technique is widely used in the multiplication of various nematophagous fungi belonging to genera *Paecilomyces*, *Pochonia*,

Arthrobotrys, *Monacrosporium*, *Dactylella* and *Dactylaria*.

For preparing the formulation in sorghum (*Sorghum bicolor* (L.) Moench), sorghum grains are soaked in water in a volume ratio of 2:1 (water:sorghum) for 12 h, excess water is decanted, and the grains are placed in polypropylene bag and autoclaved at 120°C at 1 ATM pressure for 20 min. As soon as the material cools down to the ambient temperature, it is inoculated with fungal colonies. Two discs of 15 days old fungal colonies of 8 mm, containing mycelia, are added to each substrate of 500 g. It is also recommended to add a conidia suspension ranging from 10^4 to 10^8 conidia/ml to the same substrate amount (Martinelli *et al*., 2012a). Substrate incubation period takes approximately 20 days and after that it can be used in the field (Fig. 6.1).

6.3.3 Formulation in sugarcane bagasse and rice bran

There are several reports related to the development of nematophagous fungi formulation based on sugarcane bagasse (Soares, 2006; Soares *et al*., 2007; Martinelli, 2011). Sugarcane bagasse, a by-product generated from sugar and ethanol production, is a promising substrate for the development of commercial biological products. It is inexpensive and a good source of nutrients for the fungi. When bagasse is enriched with rice bran and water, it becomes a high quality substrate for the multiplication of nematophagous fungi. The formulation is basically composed of sugarcane bagasse and rice bran in 2:1 ratio, with 200 ml of water for each litre of bagasse and rice bran mixture. The material is autoclaved at 120°C and 1 ATM pressure for 40 min. After it has cooled, the fungal isolates are inoculated. The inoculation process is performed in a laminar flow chamber in order to avoid substrate contamination. The inoculation later on results in the formation of discs of fungal colonies (Fig. 6.2).

When nematophagous fungi are multiplied in liquid media, the inoculation is performed via liquid to facilitate the inoculation process. Martinelli *et al*. (2009) determined an appropriate liquid medium for the multiplication of fungal BCAs. They reported that PDA and 1% yeast extract were favourable for the growth and sporulation of the fungal isolates (Table 6.1).

The substrate based on sugarcane bagasse after sterilization and inoculation was

Fig. 6.1. Sorghum grain substrate colonized by *Paecilomyces lilacinus* and *Pochonia chlamidosporia* ready for application in the field after 20 days of incubation.

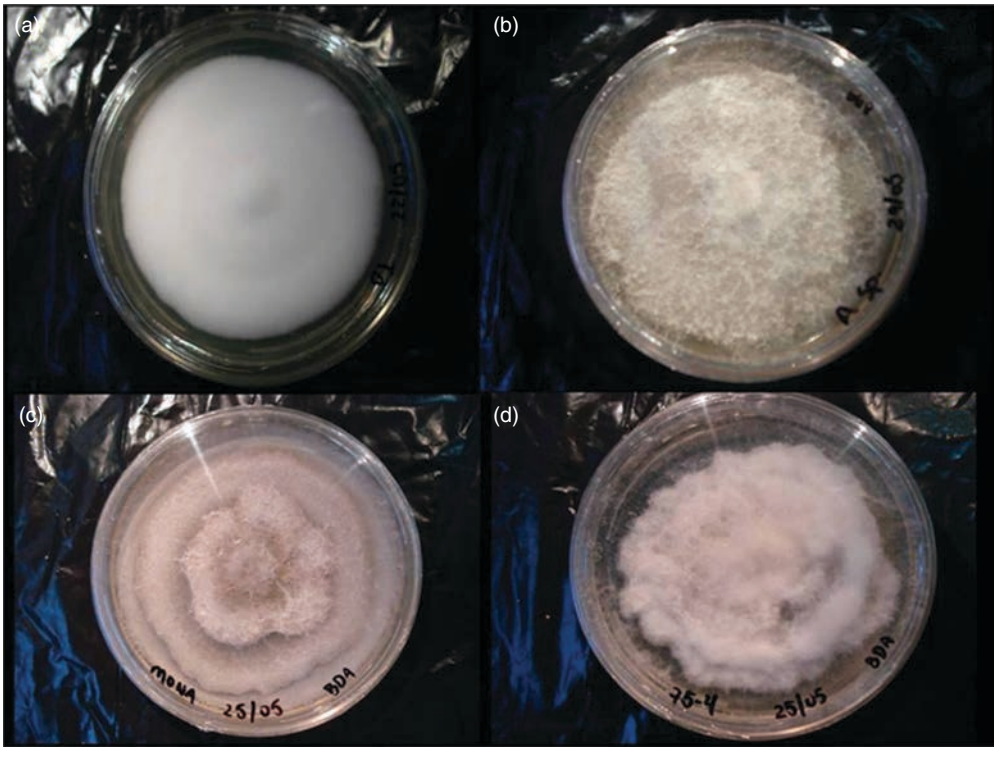

Fig. 6.2. Fungal colonies (15 days old) in PDA culture media, ready for inoculation in the substrate for the formulation of nematophagous fungi: (a) *Paecilomyces lilacinus*, (b) *Arthrobotrys oligospora*, (c) *Monacrosporium eudermatum* and (d) *Arthrobotrys robusta*.

Table 6.1. Evaluation of the colony-forming unit (CFU) of eight nematophagous fungi isolates on two different culture media kept at the mean ambient temperature of 26.5°C in the dark for 20 days.

	Evaluation of spore number on different culture media							
Culture media	AM	AO	AR	MEU	MEL	PC-10	PL	DL
PD	1.5×10^3	5.8×10^3 a	4.9×10^7 a	1.5×10^6 a	2.7×10^6 a	2.2×10^6 a	2.5×10^7 a	0.0 b
YE	1.5×10^3	1.4×10^3 b	1.5×10^5 b	2.6×10^4 b	1.4×10^4 b	4.1×10^5 b	2.1×10^5 b	4.7×10^5 a
F-test	0.42^{ns}	18.52**	19.79**	91.39**	133.73**	103.31**	562.41**	8.89*
CV %	6.01	40.03	63.17	28.58	24.20	19.29	11.72	94.82

Means followed by the same letters in the column do not statistically differ from each other by the Duncan test at 5% probability.
PD, potato and dextrose; YE, 1% yeast extract; AM, *Arthrobothrys musiformis*; AO, *Arthrobothrys oligospora*; AR, *Arthrobotrys robusta*; MEU, *Monacrosporium eudermatum*; MEL, *Monacrosporium elegans*; PC-10, *Pochonia chlamydosporia* isolate 10; PL, *Paecilomyces lilacinus*; DL, *Dactylella leptospora*.
**, significant at 1% probability;
*, significant at 5% probability; ns, non-significant.

kept at a mean temperature of 25°C in the dark. Since the Brazilian summer mean temperatures are generally ideal for growth of fungal isolates, the substrate was kept at ambient temperature (Fig. 6.3).

6.4 Cocktail Formulation

On considering parasitism/predation forms of different nematophagous fungi, researchers came up with an idea that if a formulation

Fig. 6.3. Production of the nematophagous fungi formulation based on sugarcane bagasse and rice bran. (a) Formulation prepared in sugarcane bagasse, rice bran and water; (b) substrate in polypropylene bags, autoclaved and ready for inoculation; (c) substrate colonized by *Paecilomyces lilacinus* after 10 days of incubation; and (d) chamber for nematophagous fungi growth in substrate at the ambient temperature of 25°C.

containing some nematophagous fungi with different capturing strategies is applied in the soil, the chance of success would increase. Santos and Ferraz (2000) named this mixture 'nematophagous fungi cocktail'. Initially, the cocktail was formulated using rice grains, but currently it uses a substrate based on sugarcane bagasse. For the preparation of the cocktail, nematophagous fungi are separately multiplied and after complete substrate colonization, they are mixed in equal proportion, thus resulting in the formation of a nematophagous fungi cocktail. The first cocktail formulation comprised the isolates of *P. lilacinus*, *A. musiformis*, *A. oligospora*, *D. leptospora* and *Monacrosporium robustum*, and was specifically used against root-knot nematodes, *Meloidogyne* spp. (Santos and Ferraz, 2000). Later on, Soares (2006) evaluated its efficacy in the management of PPN on some crops, chrysanthemum (*Dendranthema grandiflora*), lettuce (*Lactuca sativa*), pepper (*Capsicum annuum*) and okra (*Abelmoschus esculentus*). The formulation yielded promising results on all the crops. Martinelli (2008; Martinelli *et al*., 2012a) tested a cocktail formulation consisting fungal isolates of *M. eudermatum*, *D. leptospora*, *A. musiformis*, *A. conoides* and *P. lilacinus* against PPNs *T. semipenetrans* and *P. jaehni* on citrus plants. A significant reduction in nematode population was recorded and these results were at par with those obtained with the use of chemicals against these nematodes on citrus.

6.5 Nematophagous Fungi: Application Technology

Numerous studies have been done in different countries of the world on the biological control of phytonematodes by using nematophagous fungi (Van Gundy, 1985; Al-Hazmi *et al*., 1993; Stirling and Mani, 1995; Warrior *et al*., 1999; Soares, 2006; Martinelli, 2008, 2011; Soares and Santos, 2010; Barbosa *et al*., 2011). These studies indicate that for the application of fungal formulations under field conditions it is necessary to observe some important characteristics of the soil: the organic matter content, humidity, temperature, relative humidity and the presence of nematodes in the area. The organic matter content is related to the life stages of nematophagous fungi. According to Persson (1997), the nematophagous fungi have two phases: (i) the saprophytic phase, where the soil organic matter is employed as a source of carbon (energy) and amino acids (nitrogen); and (ii) the parasitic phase, where it is nourished by the body of captured nematodes. It has been proved that in the presence of nematodes, fungi are able to transfer themselves quickly from the saprophytic to the parasitic phase. Furthermore, the presence of nematodes causes the germination of spores and development of trapping devices. Most nematodes remain mobile throughout their life cycle and nematophagous fungi need to produce traps or adhesive hyphae to infect them. However, the sedentary nematodes such as soybean cyst nematode, *Heterodera glycines* and root-knot nematode, *Meloidogyne* spp. can be parasitized by vegetative hyphae, without the formation of specialized infective structures by the fungus.

6.5.1 Soil humidity

Availability of optimum soil humidity is extremely important for survival of nematophagous fungi and their establishment in soil. Therefore, soil humidity should always be close to field capacity. Martinelli *et al*. (2012a) correlated the average temperature, relative humidity and average rainfall in the application of nematophagous fungi for the control of *P. jaehni* in citrus orchards, but no correlation with climatic factors and survival of fungi in the soil was noticed. Kerry (1987) explained that soil humidity is seldom a limiting factor for the growth of most fungi, but it affects the dispersion of spores, especially zoospores.

6.5.2 Time of application

The application time can also be a barrier in the application of fungal BCA, especially if it coincides with the dry season and the crops are not irrigated; the survival of microorganisms depend on the humidity content of the soil. Application in warmer hours of the day needs immediate incorporation of fungal BCA into the soil, so that it may escape the harmful effects of scorching sunlight that may lead to the death of the microorganisms prior to its action (Martinelli, 2008).

6.5.3 Survival ability

Martinelli *et al*. (2012b) studied the survival ability of five nematophagous fungi (*A. robusta*, *A. oligospora*, *A. musiformis*, *D. leptospora* and *M. eudermatum*) after their application. Isolates of *D. leptospora* were found even 6 months after application in plots treated with 2 l of the formulation, but other fungal BCAs were not present at this period. Jaffee and McInnes (1991) explained that the proportion of nematodes parasitized by nematophagous fungi had correlation with the population density in the soil in peach orchards. In a laboratory experiment it was observed that the parasitism percentage was increased by nearly 100% when a high density nematode was maintained in soil but decreased to almost 0% at low densities (Jaffee, 1992). Martinelli *et al*. (2012b) monitored the survival ability of fungi for a period of 12 months after its application in citrus orchards. The survival of *Arthrobotrys* was noticed even under adverse conditions of low temperatures and soil humidity in the colder months close to the permanent wilting point. Martinelli and Santos (2010) used the same formulation containing five isolates of nematophagous fungi associated with

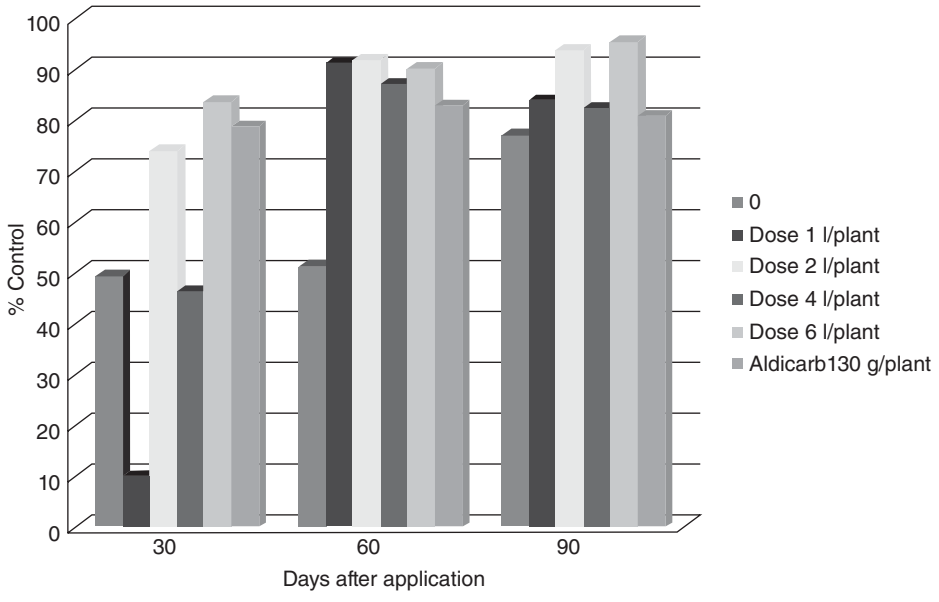

Fig. 6.4. Effect of the formulation of five nematophagous fungi (*A. robusta*, *A. oligospora*, *A. musiformis*, *Dactylella leptospora* and *Monacrosporium eudermatum*) in the management of *Tylenchulus semipenetrans* in a citrus orchard.

aldicarb for managing *P. jaehni* and *T. semipenetrans*. The study was conducted for a period of 1 year and the treatments were reapplied 180 days after first application. The results showed a significant reduction in the population of both nematode species after 180 days as compared to control. The addition of aldicarb to the formulation proved highly compatible because it reduced the population of nematodes without reducing the efficacy of nematophagous fungi. The use of such formulation (fungus + nematicide) in biological control of citrus nematode can be a substitute for chemical control, as the experiment showed that there is no incompatibility between the fungus and the nematicide. The efficacy of the formulation in relation to nematicide has also been found more economical (Fig. 6.4). Soares (2006) conducted several studies with the cocktail of nematophagous fungi on crops of chrysanthemum, lettuce, peppers and okra. These experiments showed very impressive results in controlling nematodes. It was concluded that the efficacy of nematophagous fungi is inversely proportional to the longevity of the crop; a long duration crop requires more than one application of the fungal formulation.

6.6 Conclusions

The biological control of phytonematodes has experienced a significant growth in recent years. Formulation and mass production of nematophagous fungi represents a huge market niche that incurs billions of dollars in a year, and has been awakening the interests of several companies that are producers of pesticides. However, the current experience suggests that BCAs are not going to substitute for chemical nematicides but, when applied in combination with nematicides, they can play an important role in the management of PPNs. The urgent need in reducing the dependence on chemical nematicides provides the necessary impulse to the considerable research in the field of formulation, mass production and precise application technology of nematophagous fungi.

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7 Nematophagous Fungi: Commercialization

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7.1 Introduction

It is estimated that about 842 million people, or 12% of the global population, did not have enough food to satisfy their dietary energy requirements in 2011–13. This means that approximately one in eight people in the world are likely to have suffered from chronic starvation (FAO *et al*., 2013). Plant diseases are considered a significant threat to increasing agricultural productivity since they can cause serious losses and in turn endanger food security (Strange and Scott, 2005). At least 12% of worldwide food production is lost due to plant-parasitic nematodes (PPNs) (Nicol *et al*., 2011) and this quantity is too high to be ignored. Therefore, it becomes mandatory to decrease the level of damage caused by PPNs to agricultural and horticultural crops, though it is a daunting task and difficult to achieve. Management of PPNs has been principally based on application of chemical nematicides but there is a need to substitute chemicals with other effective methods. The efficient synthetic nematicides are not affordable by a lot of growers or have generally been taken off the market due to concerns about the environmental hazards and human health (Davies and Spiegel, 2011a,b; Moosavi, 2012; Moosavi and Zare, 2012; Quesada- Moraga *et al*., 2014).

Biological control is considered an appropriate alternative to chemicals since it is not only an environment-friendly measure but also can promote sustainability in agricultural production (Askary, 2010; Stewart *et al*., 2010; Popp *et al*., 2014). Illustrating that selected biocontrol agents (BCAs) may provide sufficient control level along with political pressures for disease management programmes that do not depend on chemicals, have all contributed to a modification in attitudes toward investigation into biological control (Fravel, 2005). Numerous organisms have shown antagonistic activity against PPNs (van der Putten *et al*., 2006; Costa *et al*., 2011; Stirling, 2011) and among them fungi are considered the most important group (Hallmann *et al*., 2009; Timper, 2011; Moosavi and Zare, 2012; Pendse *et al*., 2013). These organisms mostly have not provided consistent or adequate control. However, the best results for biological control of soil-borne pathogens can be achieved when short-term protection would cause considerable yield benefits and where inundative treatments of target sites are possible (Deacon, 1991).

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Many fungi from different taxonomic groups can adversely affect PPNs (Moosavi and Zare, 2012), however possessing aggressiveness is not the only required characteristic for being a qualified BCA. They must meet a wide range of desirable traits, which should only be justified after many interconnected successive experiments. The ultimate objective of biocontrol investigation is to present extra tools for disease management. To deliver these tools to growers, potent BCAs must be commercialized (Wilson and Jackson, 2013). The present chapter is focused on the commercialization processes that a fungal BCA has to pass before it could be commercially introduced into the market.

7.2 Nematophagous Fungi

Nematode antagonistic fungi can naturally infect one or more stages of living nematodes (eggs, juveniles and adults) for their nutrition and decrease the population densities of nematodes. These fungi include more than 200 species of taxonomically different groups, which can be classified into nematophagous fungi and endophytic fungi. Nematophagous fungi are further subdivided into egg- and female- parasitic fungi, nematode-trapping fungi, endoparasitic fungi and toxin-producing fungi (Moosavi and Zare, 2012). The taxonomy of nematode antagonistic fungi as well as their mode of action is briefly shown in Table 7.1.

Nematode antagonistic fungi have drawn a lot of attention so far partly due to their high adverse activities against both plant and animal parasitic nematodes and partly due to their amazing morphological adaptations in hunting and parasitizing nematodes (Askary, 1996; Nordbring-Hertz *et al*., 2011).

7.3 Current Status and Challenges

The primary researches on nematode antagonists were mainly descriptive, which resulted in the isolation and identification of many nematode natural enemies, but the first attempt to use nematophagous fungi as biological control was in the late 1930s (Linford, 1937). However, the real attempts to replace chemical nematicides with bioproducts began in 1977 with the loss of DBCP (Ferris *et al*., 1992). Theoretically, it was supposable to introduce mass propagated antagonists into soil or establish them on seeds or roots and achieve an acceptable level of control. Thus, many research groups started the screening processes to search for microorganisms with potential for biocontrol of nematodes (Stirling, 2011).

Initial studies by academic scientists created a strong hope for finding a sustainable control solution safe to both the environment and humans. Entrepreneurs have tried to develop and commercialize these research results into viable products but in practice only a few bioproducts have successfully been introduced to the market. A list of fungal commercial products for the management of PPNs is given in Table 7.2.

This limited success can be attributed to many technical problems as well as insufficient knowledge on the commercialization process. The main impediments are the: inability to mass produce commercially accepted formulations for field implementations; low and variable aptitude of introduced fungi to become established and remain active in complex soil environment; and their high prices (Kerry, 2000; Jansson and Lopez-Llorca, 2004).

In fact, it is now hard to claim that the early expectations for the strength of bionematicides on solving the agricultural problems have materialized. However, it would also be a mistake to deduce that biological control cannot be developed to provide a reliable and effective control measure. Existence of suppressive soil demonstrates that BCAs can certainly generate robust and durable nematode control, but the contributing factors have not completely been identified yet. Recognizing these factors, scientists should assist in modifying farming systems to employ and enhance such suppressive mechanisms (Stirling, 2011). Furthermore, a better understanding of the infection processes of PPNs by fungal BCAs could result in the progress of more effective and dependable control (Tunlid and Ahrén, 2001; Morton *et al*., 2004; Davies, 2005). Teamwork among several disciplines

Table 7.1. Taxonomy of some nematode antagonistic fungi and their infection mechanism.

Fungal group	Phylum	Teleomorph	Anamorph	Mode of parasitism
Nematophagous fungi				
Egg- and female-parasitic	Oomycota	*Nematophthora*		Zoospores
	Ascomycota	*Metaordyceps*	*Pochonia*	Appressoria
		Cordyceps	*Purpureocillium*	Appressoria
		Cordyceps	*Lecanicillium*	Appressoria
Nematode-trapping	Zygomycota	*Stylopage*		Adhesive hyphae
		Cystopage		Adhesive hyphae
	Ascomycota	*Orbilia*	*Arthrobotrys*	Adhesive networks
		Orbilia	*Dactylellina*	Adhesive knobs and/or non-constricting rings
		Orbilia	*Drechslerella*	Constricting rings
		Orbilia	*Gamsylella*	Adhesive branches or unstalked knobs
	Basidiomycota	*Hohenbuehelia*	*Nematoctonus*	Adhesive 'hour-glass' knobs
Endoparasitic	Oomycota	*Myzocytiopsis*		Zoospores
	Chytridiomycota	*Haptoglossa*		'Gun cells', injection
	Blastocladiomycota	*Catenaria*		Zoospores
	Ascomycota	*Podocrella*	*Harposporium*	Ingested conidia
		?	*Drechmeria*	Adhesive conidia
		Cordyceps?	*Haptocillium*	Adhesive conidia
		?	*Hirsutella*	Adhesive conidia
	Basidiomycota	*Hohenbuehelia*	*Nematoctonus*	Adhesive spores
Toxin-producing	Basidiomycota	*Pleurotus*		Toxic droplets
		Coprinus		Toxin, 'spiny structures'
	Ascomycota	?	*Myrothecium*	Secrete toxin
Endophytic fungi				
Mycorrhizal fungi	Glomeromycota	*Glomus*		Unknown[a]
		Gigaspora		
Neotyphodium endophytes	Ascomycota	Several genera	*Neotyphodium* spp.	Unknown[a]
Fusarium endophytes	Ascomycota	Several genera	Non-pathogenic *Fusarium* species	Unknown[a]

[a]Endophytic fungi may use one or a combination of several mechanisms against plant-parasitic nematodes.

Table 7.2. Commercial products of fungal biocontrol agent in the management of phytonematodes.

Fungus	Product	Formulation	Company/institution	Country
Pochonia chlamydosporia	KlamiC®	Granulate	Rothamsted Research and Centro Nacional de Sanidad Agropecuaria	UK Cuba
P. chlamydosporia	PcMR-1 strain	Liquid	Clamitec, Myco solutions, lda	Portugal
P. chlamydosporia	Xianchongbike	Liquid	Laboratory for Conservation and Utilization of Bio-resources, Yunnan University	China
Purpureocillium lilacinus	BIOACT®WG	Water-dispersible granulate	Bayer Crop Science	USA
P. lilacinus	BIOACT®WP	Water-dispersible powder	Bayer Crop Science	USA
P. lilacinus	PL Gold	Wettable powder	BASF Worldwide, Becker Underwood	South Africa
P. lilacinus	PL 251	Water-dispersible granulate	Biological Control Products	South Africa
P. lilacinus	BIOCON	Wettable powder	Asiatic Technologies Incorporation	Philippines
P. lilacinus	Shakti Paecil	Wettable powder	Shakti Biotech	India
P. lilacinus	Yorker	Wettable powder	Agriland Biotech	India
P. lilacinus	Miexianning	Unknown	Agricultural Institute, Yunan Academy of Tobacco Science	China
P. lilacinus	PI Plus® (*P. lilacinus* strain 251)	Wettable powder	Biological Control Products	South Africa
P. lilacinus	Melocon®WG	Water-dispersible granulate	Prophyta GmbH Certis	Germany USA
Trichoderma harzianum (isolate DB 104)	Romulus	Wettable powder	Dagutat Biolab	South Africa
Myrothecium verrucaria	DiTera®	Dry flowable	Valent Biosciences Corporation	USA
T. harzianum IIHR-Th-2	Ecosom-TH	Wettable powder, liquid, lyophilized	Agri Life SOM Phytopharma (India) Limited	India
T. harzianum	Commander Fungicide	Unknown	HTC Impex Private Limited	India
T. harzianum	Trichobiol	Wettable powder	Control Biológico Integrado; Mora Jaramillo Arturo Orlando – Biocontrol	Colombia
T. viride (strain 2684)	Trifesol	Wettable powder	BioCultivos S.A., Bogotá, Colômbia	Colombia
T. lignorum	Mycobac	Unknown	Laboratórios Laverlam	Colombia
Myrothecium verrucaria	DiTera®	Dry flowable	Valent Biosciences Corporation	USA

such as nematology, plant pathology, soil ecology, agronomy, biochemistry and molecular biology is required to comprehend more clearly the biotic interactions in the rhizosphere. This collective information should surely boost the probability of successful implementation of fungal BCAs as a reliable control measure (Stirling, 2011; Tunlid and Ahrén, 2011). In addition, the genome of fungal BCAs can be manipulated to increase their activity against PPNs and/or enhance their survival in soil.

The same regulations are applied to chemical pesticides and biological ones in spite of their fundamental differences. Also the commercialization and registration process of biological products is adapted from chemical pesticides (Gaugler, 1997; Harman *et al*., 2010). The developmental model for chemicals is based on inexpensive and reliable products that can easily be scaled up and used for major crops, but BCAs hardly match this model. Biopesticides poorly possess the three important characteristics that are prized by growers: low price, high and stable performance and ease of use (Gaugler, 1997). Registration also needs to be modified in order to be more compatible with biological products. A complete registration process requires plenty of time, money and effort to accomplish toxicological, ecological and sometimes efficacy assays (Harman *et al*., 2010). There is an obvious need to devise a detailed and reliable roadmap to the successful development and commercialization of microbial BCAs (Ravensberg, 2011b).

7.4 Development and Commercialization Process

Hypothetically in modern agriculture, microbial control agents including fungi present one of the most sustainable and ecofriendly methods for PPN management. The approximate annual growth of biopesticide sales during the last 10 years has been 10% (Ravensberg, 2011a), though it is estimated that this will increase up to 15.6% in 2014 (Regnault-Roger, 2012). Only a scant share (3.5%) of the global pesticide market in 2009 belonged to biopesticides, which was equal to US$1.6 billion in monetary terms (Lehr, 2010). Though the main part of the biopesticide market is allocated to insect control, other biocontrol products are also on the market with activity against fungi, weeds and nematodes (Wilson and Jackson, 2013). There is increasing attention in commercialization of BCAs and many companies, including multinational agrochemical companies, have enhanced their investment in research and development of such products (Fravel, 2005).

Practical researches on BCAs against PPNs initiated with the isolation and identification of antagonistic organisms. The next steps continued with soil environmental manipulation to enhance antagonism, defining their mode of action and development of qualified organisms to commercial products (Hallmann *et al*., 2009). Notwithstanding the identification of numerous organisms in these extensive investigations, only a few of them are now available in the market and their application in management of PPNs is yet marginal. This causes the scientists to search carefully for hurdles and restrictions coming in the way of development of a BCA as a commercial product. The main reasons for failure are designated as: inconsistency of their efficacy; variable quality of biocontrol products; their expense contrasted to their effectiveness; difficulties in their scale-up production, formulation, stabilization and delivery system; registration costs; overestimating the size of target markets; failure in launching a new product into the market; inaccurate assessment of the overall needed budget and the time to market; and inability to achieve the company's financial objectives (Whipps and Davies, 2000; Whipps and Lumsden, 2001; Dong and Zhang, 2006; Timper, 2011). The development of a BCA to a viable commercial product is only possible if developing processes are compatible with industrial and commercial development methods and if the formulated products are suitable for field implementation (Lumsden *et al*., 1995).

A vast majority of the work on biological control of PPNs has been performed on isolation, identification and development of a BCA and only a little on market and commercial aspects, and even less on economic considerations. This elucidates the reason for failure of

many promising BCAs in a successful launch on the market, and also the reason for failure of numerous companies in the establishment of a profitable business. History of the biopesticide business shows that many potent BCAs remain on the shelf of academic scientists without being commercialized, mainly because of paying inadequate attention to economic aspects and market considerations. Participation of industry research and development (R&D) is required to support and accelerate the development and registration of more bioproducts in an economical way. In addition, close cooperation of public and private organizations is needed to instruct farmers, retailers and society on the merits of bioproducts.

Total reappraisal of the commercialization procedure emphasized that successful products may be developed only if a rational and systematic pathway is followed by a multidisciplinary team of researchers. Project management is an essential tool to prevent time and money being wasted in this complex pathway (Ravensberg, 2011b). Here, the commercialization processes of a nematode BCA are concisely described in a structured manner; however, the detailed information is available in other chapters of this volume.

7.4.1 Selection of biocontrol agents

The first step in the selection programme of BCAs is defining a nematode problem that cannot be controlled by routine measures. The problem must be big enough to ensure an adequate market size and consequently to justify development costs in terms of money and time. The next successive steps are seeking for a solution, collecting potential BCAs and choosing the best species or isolate according to a well-defined selection process.

When the problem is characterized in detail, the most important step in the process of developing a commercial bionematicide is the investigation for and the selection of a qualified agent. The final success of a bioproduct is greatly related to how well the searching and screening process is carried out (Fravel, 2005). Finding organisms with biocontrol activity against plant pests can be achieved by three main ways: (i) inspection from basic research; (ii) screening a variety of organisms against an existing problem (Tormala, 1995); and (iii) selecting suppressive organisms to PPNs from agricultural soils (Kerry and Hominick, 2002; Costa *et al*., 2011; Timper, 2011). Two kinds of pest suppression exist in agricultural soils: general or non-specific suppressiveness and specific suppressiveness (Stirling, 2011). Just a scant proportion of nematodes (less than 10%) are able to complete their life cycle in nematode-suppressive soils and reproduce the next generation (Kerry and Crump, 1977). There are several techniques by which it is determined whether a soil includes antagonistic organisms against PPNs or not (Westphal, 2005; Borneman and Becker, 2007), however identifying the responsible organisms for nematode suppression are usually too difficult. An entirely different spectrum of microorganisms is frequently involved in preventing multiplication of nematode populations. Among the fungi, usually identified are *Pochonia chlamydosporia*, *Trichoderma* spp., *Dactylella oviparasitica*, *Hirsutella rhossiliensis* or *Fusarium* spp. whereas the bacteria involved are identified as *Pasteuria penetrans*, *Bacillus* spp. and *Pseudomonas* (Davies and Spiegel, 2011c).

Stepwise processes to instruct the selection of isolates with biocontrol activity against plant-pathogenic fungi and bacteria need to be developed. Such protocols will be helpful to select the more efficient, ecofriendly, cost-effective and safe isolates (Kohl *et al*., 2011). Careful selection of BCAs against PPNs consists of several levels. The first step is the choice of the agent type, i.e. whether the nematode host is migratory or sedentary and also whether the strategy is to decrease the damage caused by the nematode or its reproduction. Evaluation of the pathogenicity and virulence against the target nematodes along with assessment of growth in rhizosphere and determination of its nutritional and ecological (temperature, pH, moisture, etc.) optima are the next levels (Kerry and Hominick, 2002). Various fungal isolates differ greatly in their abilities to control nematodes, to survive (Moosavi *et al*., 2010, 2011), to tolerate abiotic stresses and to increase plant performance and yield (Harman *et al*., 2010). Therefore, it is imperative to examine the maximum possible number of isolates

during screening programmes. Sometimes it is necessary to evaluate hundreds to thousands of isolates to encounter a few with an appropriate combination of characteristics for biocontrol (Glare *et al*., 2012). The development of novel fungal-based bioproducts relies on the availability of proper isolates. Only a scant proportion of candidate isolates (less than 1%) may finally succeed to reach market (Bailey and Falk, 2011). Assessment of pathogenicity against PPNs is carried out by several methods (Irving and Kerry, 1986; Gunasekera *et al*., 2000; Lopez-Llorca *et al*., 2002), but it is not an easy task (Jansson and Lopez-Llorca, 2004). In certain circumstances, the changing of favourable host may take place even at kingdom level (Nikoh and Fukatsu, 2000; Lopez-Llorca and Jansson, 2007; Moosavi *et al*., 2011).

The necessity of using host-plant at early phases of the selection process will extend the time and enhance the expenses of choosing potential candidates. In addition to aggressiveness to PPNs, several other factors are determinant in selecting potent isolates. These are mass productivity, wider host range, production of resting structures and safe to non-target organisms (Kerry and Hominick, 2002). Although narrow host-range makes the BCAs safer to the environment and non-target organisms, it also restricts the market size to niche markets, which in turn makes development and commercialization uneconomical. Soil receptivity to selected propagules of nematophagous fungi must also be determined prior to any further investigation on formulation or scale-up production (Wakelin *et al*., 1999).

At this point of the process and according to gathered information on BCA qualification, demand for the product, potential market size and on existing competing products, a critical decision must be made as whether to continue with the development of the BCA or not. Any further investigations should be stopped on futureless isolates to prevent more loss of time and money.

Protecting the intellectual property rights (product or technological idea) is also a very important subject if the producer decides to continue the development of a BCA. The success of the biopesticide business is not ensured if the owner is unable to receive exclusive rights to his developed BCA (Montesinos, 2003). Patent protection will be helpful here, however it is a costly and time-consuming process. Moreover, the strength of patenting is increasingly challenged by genetic engineering (Tormala, 1995; Ravensberg, 2011c).

7.4.2 Mass production

One principal characteristic for a fungal BCA which is intended to be developed as a biocontrol product is mass productivity. The objective of mass production is to economically generate the utmost amount of effective propagules (chlamydospores, conidia, microsclerotia) in the shortest period of time. A higher cost of production is a limiting factor in commercialization, which can be a consequence of expensive substrate, low biomass output, or restricted economies of scale (Fravel, 2005). Biomass production of a number of microorganisms is troublesome or uneconomic due to specific environmental and nutritional conditions needed for their growth (Jones and Burges, 1998).

Mass production techniques for microorganisms usually comprise submerged liquid fermentation, solid-substrate fermentation and a two-phased system (Ravensberg, 2011c). Fungal BCAs typically produce a uniform cell culture in liquid state fermentation, but oxygen supply has to be maintained. Solid state fermentation has better aeration, but potentially is more prone to being contaminated with undesirable organisms. Mass production of fungal BCA in the two-phased system initiates with production of BCA in a liquid culture and continues with sporulation in a solid phase (Wraight *et al*., 2001). However, it is also possible to mass produce fungi on two-stage solid media with different characteristics (Sun *et al*., 2009).

It is very important to know from the very beginning whether a certain BCA is mass producible or not. Though this information is available for many known BCAs, new candidates must be examined to see if they can produce enough infective propagules on a low-cost medium. Obviously, a promising candidate is the one that can produce numerous resistant

propagules with an acceptable shelf-life and applicable with routine agricultural machinery (Yang and del Rio, 2002). Resistant spores are the best propagule for fungal BCAs that may be produced on artificial media. However, production and formulation of fungi are more difficult and more costly compared with bacteria. Virulent BCAs with troublesome mass productivity should be discarded at the beginning of the selection procedure (Ravensberg, 2011c).

The majority of nematophagous fungi are facultative parasites and therefore are growable on solid media or in liquid fermentation, however high numbers of resistant spores such as chlamydospores are rarely produced in submerged culture. Resistant structures are easily formulated and can facilitate BCA handling. In addition, they can provide longer shelf life. Fungal BCAs that are unable to develop resting structures may be applied as vegetative cells, hyphae or conidia. These structures are more vulnerable and need more complex formulations (Kerry and Hominick, 2002). There are several methods for producing propagules of a certain fungal BCA, however, methods that produce the highest number of propagules are not essentially those that generate the most efficacious or the best type of propagule (Brannen and Kenney, 1997; Jackson, 1997).

We have adequate knowledge about the growth requirements of some BCAs to force them to produce the desired propagules. The important factors in obligating fungi to produce specific propagules include carbon source, osmotic potential, temperature and pH (Fravel, 2005). For instance, stimulation of conidia production in *Trichoderma* sp. occurs by modifying the carbon source (Agosin and Aguilera, 1998) and the propagules with the longest shelf life are produced when the C:N ratio is 14:1 (Engelkes *et al*., 1997). Compared with the traditional continuous method, sporulation of *P. chlamydosporia* was the highest when it was grown on the 'two-stage' solid media with different carbon concentrations and C:N ratios (Gao and Liu, 2010), but sporulation of *Purpureocillium lilacinus* showed no difference between traditional and new methods (Sun *et al*., 2009).

7.4.3 Formulation and application

Successful establishment of BCAs in soil as well as persistence of their populations over a threshold are the prerequisites to provide an efficient biological control. Concerning the heavy weight of soil (2500 t/ha for the upper 25 cm) that needs to be inoculated sufficiently with efficient dose of BCA (10^3–10^6/g soil), it appears that broadcast treatment for control of PPNs is probably uneconomic (Kerry, 1998; Sikora *et al*., 2008). Therefore, controlled and restricted placement of inoculum is proposed via a proper formulation (Kerry and Hominick, 2002). Delivering an optimal population density of BCA at the most appropriate site and time is the main goal of formulation (Warrior *et al*., 2002). Formulation is the process in which selected BCAs (active ingredients) are combined with a variety of additives (a suitable carrier and added adjuvant) to produce a final marketable product. Formulation process of biopesticides has been reviewed in several articles (Burges, 1998; Fravel *et al*., 1998; Wraight *et al*., 2001; Warrior *et al*., 2002; CPL, 2006; Askary, 2010; Ravensberg, 2011c), however formulation details are sometimes considered as trade secrets and accordingly are not generally accessible (Fravel, 2005). A proper formulation could improve the efficiency by four ways. These include: (i) stabilization of the BCA throughout production, distribution and storage; (ii) facilitation of handling and utilization of the product and consequently improvement in the delivery time of active ingredient to the target organisms in the best form and manner; (iii) enhancing persistence of the BCA by preserving it from unfavourable environments; and (iv) increasing bioactivity at target site via improving BCA's activity, rate of release, multiplication, contact and interaction with the target organism (Jones and Burges, 1998). Optimizing the formulation to all these objectives is not always feasible, thus formulation usually is a compromise between these goals. Most bionematicides in the market are in the form of liquid or wettable powder formulations (Table 7.2), which are implemented in furrow or through drip irrigation systems (Davies and Spiegel, 2011c). The mode of action of

BCA as well as type of propagule influences formulation choices (Ravensberg, 2011c).

Arthrobotrys oligospora is one of the most studied fungi among nematode-trapping fungi which possesses several advantages like fast growing capability, being able to culture on artificial media and capable of colonizing the soil and rhizosphere (Singh *et al*., 2013). A product of other species of this genus has been marketed. *Arthrobotrys robusta* and *Arthrobotrys irregularis* have been used in France with the trade name 'Royal 300' (Cayrol *et al*., 1978) and 'Royal 350' (Cayrol, 1981, 1983), respectively. They were applied as fresh mycelium on organic substrates, but could not generate consistent control. Moreover, their inocula were difficult to handle. Alginate-based formulations of *Monacrosporium ellipsosporum* and *Monacrosporium cionopagum* have been produced on a small scale (Jaffee and Muldoon, 1995) and the activity of nematode-trapping fungi, *Dactylella candida* and *Arthrobotrys dactyloides*, were examined following their encapsulation in alginate (Stirling and Mani, 1995). *A. dactyloides* formulated in kaolin, vermiculite and gum arabic granules obtained from liquid fermentation had a poor survival ability, however its persistence was greatly enhanced when a limited solid-state fermentation was added to the process (Stirling *et al*., 1998b). The hope of using nematode-trapping fungi in biological control of PPNs can be regenerated by our enhanced knowledge on their bio-ecology and improved techniques in their formulation and application to soil.

Pochonia chlamydosporia can be formulated on alginate (Kerry *et al*., 1993) and kaolin (Stirling *et al*., 1998a). The fungus has also been mass produced in submerged fermentation and the biomass transformed into a granular product (Stirling *et al*., 1998a). A granulate formulation of this fungus has been commercialized in Cuba by the trade name of KlamiC®, which is applied as soil incorporation (Davies and Spiegel, 2011c). *P. chlamydosporia* does not produce chlamydospores in liquid culture and the spores are obtained from solid culture (see Manzanilla-Lopez *et al*., 2013). *Purpureocillium lilacinus* has been developed to a wettable powder (Melancon®, Bioact®) or Suspo-emulsion (Yorker®) commercial product for use in vegetables and fruit trees against cyst and root-knot nematodes (Davies and Spiegel, 2011c). A granular formulation based on alginate has been developed for *H. rhossiliensis* (Lackey *et al*., 1993); fermentation and microencapsulation of this fungus in hollow beads enhanced fungal growth as compared to alginate beads (Patel *et al*., 2011). *Myrothecium verurrucaria* is a toxin-producing fungus and its nematicidal product is developed either as a water-dispersible granule (WDG) for use in turf and ornamentals or as an oil-based emulsifiable suspension (ES) for use in grapes (Warrior *et al*., 1999, 2002; Wilson and Jackson, 2013).

An appropriate delivery system can significantly reduce the excess amount of BCA inoculum. The delivery technology should be devised depending on BCA type, mode of action and cropping scheme (Fravel and Engelkes, 1994; Fravel, 2005), therefore it is better to apply BCA formulations with the help of standard available agricultural equipment, otherwise acceptance of BCA by growers is improbable. Similar to other businesses, one important consideration in industrial production of BCAs is devising logical quality control (QC) steps. If working with living organisms with the lack of stringent guidelines, it will be hard to obtain uniform bioproducts with good and stable quality standards (Desai *et al*., 2002). QC refers to production control, process control and final product control, which supervises and guarantees the purity of BCA, sufficient amounts of viable inoculum, technical characteristics and efficiency until the end of claimed shelf-life (Khetan, 2001; Ravensberg, 2011d). Poor QC consequently results in poor quality products and insufficient control. It is a general threat to biocontrol reputation and therefore QC must cover all producing processes to ensure high quality final products.

7.4.4 Legal and commercial aspects

Handling and implementation of BCAs are subjected to several regulations due to the

possibility that BCAs can cause adverse effects on both human health and the environment. Therefore, microbial BCAs have to be registered as a plant protection product before being introduced into the market (Whipps and Davies, 2000; Desai *et al*., 2002; Ehlers, 2010, 2011; Ravensberg, 2011c).

Though registration procedures are different in various countries, all of them need a large data package to accomplish data requirements. Toxicology and environmental fate are the two most essential data for registration. Once a registration dossier is submitted, it is evaluated by competent authorities to decide whether that bioproduct can be introduced to the market or not (Cook *et al*., 1996; Fravel, 2005). Accomplishing an entire registration procedure is considered as the greatest challenge in the commercialization process since it needs a lot of time, expense and effort (Ravensberg, 2011c). In spite of fundamental differences between biological and chemical pesticides, both have to pass a similar process for registration. Thus, it is essential to modify regulations in order to facilitate the development of novel bioproducts (Glare *et al*., 2012).

Besides efficiency and consistency, bioproducts must be competitive on price. The high expense of mass production, formulation and/or application has impeded many products from reaching the market place. Novel methodological or technological advances causing decreases in production cost are essential to reduce the end-user price. The main common mistake of unsuccessful biological-based business is overestimation of the potential market size and the foreseen market adoption rate. Launching a product successfully in the market is the last and most significant step in the entire process. To this point of developing a bioproduct, a company has to spend a lot of time and money without having any income. Thus the company will enter bankruptcy if the sale of candidate biopesticide does not supply enough revenue to make the business profitable. The potential market size and the approximate sales volume must be accurately projected as early as possible (Tormala, 1995; Ravensberg, 2011a). The nature of a biopesticide business does not allow quick profit-making and it may take several years for a company to arrive at a sales amount that would provide favourable earnings. Thus inaccurate estimation of required time and money will surely result in failure (Menzler-Hokkanen, 2006). Devising a perfect business plan at the start of the project, convincing farmers to use bioproducts and consumers to use organic products, having a capable multidisciplinary team for developing and marketing the bioproduct, and using efficient distribution strategies are crucial in the final success of a bioproduct in the market.

7.5 Efficacy of Fungal BCAs and its Effect on Commercialization

Several factors determine the efficacy of fungal BCAs, especially when they are applied in large scale conditions. These factors may be related to processes in pre-, post- and during application of BCAs. Even after efficient nematode control in small-scale experiments, many BCAs cannot suppress nematode populations under field conditions (Whipps and Davies, 2000). Many intrinsic and ecological factors may be involved in regulation of BCA performance. Only a virulent nematopathogenic BCA with high competitive saprophytic ability could produce an acceptable control level. BCAs should efficiently colonize the rhizosphere and endure a wide range of environmental conditions (Jackson and O'Callaghan, 1997; Desai *et al*., 2002). Secreting extracellular products is an important trait. For example, nematotoxic substances enhance virulence and antibiotic production increases stability in soil (Timper, 2011). Isolated BCAs are screened for these intrinsic factors and can be discarded if they do not meet these prerequisites.

Many abiotic and biotic factors affect the longevity and performance of nematode-antagonistic BCAs. Soil temperature, type, moisture, pH and nutritional status are the most important abiotic factors that influence efficacy of BCA, while the biotic factors are soil biota, host plant and nematode target (Dong and Zhang, 2006). There is insufficient knowledge of biotic factors affecting performance,

persistence and dissemination of BCAs (Kerry and Hominick, 2002), mainly as a result of difficulty in distinguishing specific biotic influences (Stewart *et al*., 2010). However, better understanding of these interactions may have a great effect on increasing the chance of bioproduct success. Being active in a wide range of environmental conditions, surviving the application process, easy and long-lasting establishment in soil as well as efficient contenders against other soil microbiota are required characteristics for developing a candidate BCA to a successful bioproduct.

Economical mass producibility, stable and persistent formulation, easy applicability and high quality products are other favourable traits for a fungus for its development as a commercial bioproduct. Production of robust survival structures will be supportive in persistence of a fungal BCA in soil as well as to facilitate the generation of a formulated product with longer shelf-life (Whipps and Lumsden, 2001; Ravensberg, 2011a; Timper, 2011). The most frequent complaint about bioproducts is inconsistency in their performance. It may be due to losing aggressiveness (Lohmann *et al*., 1989; Zuckerman *et al*., 1989; Wang *et al*., 2003), uncontrollable condition of large-scale implementation (Dong and Zhang, 2006) or more probably paying insufficient attention to quality control during pre-application steps (Jenkins and Grzywacz, 2000; Ravensberg, 2011d). Producing a high quality product with stable performance is pivotal for success at the marketing level.

7.6 Conclusions

Existence of nematode-suppressive soils emphasizes the probability of biological control as a viable method (Stirling, 2011), however the commercial use of BCAs may be lagged until suppression mechanisms are adequately understood (Davies and Spiegel, 2011c) and a structured guideline is followed for commercialization (Ravensberg, 2011b). All the steps of the developmental process must be extensively considered if a BCA is to be successfully introduced into the market. Until now, the majority of researchers have focused on isolation and selection of new isolates while other areas such as large-scale performance, durability, formulation, industrial production, delivery, marketing, distribution channels and implementation have received less attention. The areas which are supposed to cause transformational advances are enhanced efficacy, delivery and persistence (Glare *et al*., 2012).

Re-evaluation of the commercialization processes have led to a rational and systematic pathway. The development process is not a simple stepwise linear one and simultaneous working is required to save time (Ravensberg, 2011b). The development of BCAs starts with screening for and selecting qualified fungi with biological activity against PPNs (Timper, 2011). Economical mass producibility of effective propagules is an essential trait for a candidate fungus to be commercialized (Kerry and Hominick, 2002). A proper formulation assists in delivering an optimal population density of BCA at the most appropriate site and time as well as improving the efficiency (Davies and Spiegel, 2011c). Registration is the most important challenge in the commercialization process that must be overcome (Whipps and Davies, 2000). Allocating a reasonable and competitive price that can cover the expenses and make a reasonable profit is vital. Marketing is the last and most important step in the entire process and therefore a good knowledge of the market is needed (Ravensberg, 2011a). It can be concluded that future scientific and technological developments will be more helpful to overcome existing obstacles and to design a bright future for bionematicides with many new products in the market.

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8 Nematophagous Fungi: Regulations and Safety

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8.1 Introduction

Biocontrol of phytonematodes involves the use of biopesticides (also known as biocontrol agents; BCA), which, in the case of nematodes, are mainly their natural fungal predators (Stirling and Smith, 1998). Historically, phytonematodes were controlled using soil chemical fumigants such as methyl bromide, dazomet, 1,3-dichloropropene, telone, metam sodium and chloropicrin (Bell e*t al*., 1998; Sardanelli and Elision, 2005). However, recently there has been a shift from chemical control to biological methods of controlling nematodes due to several reasons. These include general awareness of the environmental pollution aspects associated with chemical control and banning of the use of methyl bromide (Chaves, 2003) and other organochlorides implicated in the depletion of the ozone layer (Sikora, 2002). The ability of fungi to control phytonematodes has been known for over a century, however the impetus to use them commercially only became significantly noticeable in the 1980s (Tunlid and Ahrén, 2011). A biopesticide can be defined as a biologically derived agent for the management of pests (Evans, 2003). However, the definition can vary according to a given country or organization (Chandler *et al*., 2011). For instance, in the USA, the Environmental Protection Agency (EPA) states that the definition includes 'naturally occurring substances that control pests (biochemical pesticides), microorganisms that control pests (microbial pesticides), and pesticidal substances produced by plants containing added genetic material (plant-incorporated protectants) or PIPs'. Biopesticides such as fungi when used in plant protection for the control of nematodes fall in the category of 'microbial pesticides'. Although the definition of microbial pesticides may differ with location, basically they are a heterogeneous group consisting of microorganisms, i.e. bacteria, fungi, viruses or protozoa, as the control agents. As crop protection products, biopesticides are often used in an augmentative role as part of an integrated pest management system (Chandler *et al*., 2011) or as an alternative to chemicals in organic farming (Wozniak, 2003).

The use of biopesticides such as fungi in most cases is regarded as being safer to humans and the environment than the chemical agents previously used. Biopesticides may include live cells or their products, however in most definitions only living entities are considered as microbials. Strains of fungal species that occur naturally in soil and which

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specialize in predation of nematodes are exploited for this task. To achieve this, they need to be cultivated and artificially applied to crops. The very nature of these fungi, i.e. soil-dwelling, nematode-specific and often widespread, makes them ideal candidates for use as biopesticides. The fungi used in nematode control employ different modes of action ranging from nematode-trapping as in *Arthrobotrys oligospora* and *Dactylaria candida* to endoparasitic as in *Catenaria anguillulae* and *Hirsutella rhossiliensis* to egg and female parasitic such as *Pochonia chlamydosporia* and *Paecilomyces lilacinus* and toxin producing such as *Coprinus comatus* and *Pleurotus ostreatus*. Therefore, these fungi may be referred to as nematicidal fungi or nematophagous fungi.

In the present chapter, our attempt is to provide some recent valuable information regarding the regulatory and safety measures, required in the use of nematophagous fungi. Limitations in the handling of fungal biopesticides and criteria for their registration at commercial level have also been discussed.

8.2 Fungi Considered Safe for Use Against Phytonematodes

The use of fungi that have been documented to be pathogens of plants, animals or insects to control phytonematodes should be avoided because they may spread to non-target organisms causing catastrophic consequences. However, there are many soil-dwelling, nematode-specific and widespread nematophagous fungi that can be exploited. Among the fungi that are considered safe for use in the control of phytonematodes are the following: *Arthrobotrys* spp., *Aspergillus fumigatus*, *Dactylella* spp., *Dechemeria* spp., *Dendriphiopsis* sp., *Fusarium chlamydosporium*, *Myzocytium* spp., *Nematoctonus* spp., *Nematophora* sp., *P. lilacinus* (most commonly used marketed as '*Pl* plus' in South Africa and Botswana), *Penicillium* sp., *Trichoderma* spp. and *Verticillium chlamydosporium*. Others such as *Alternaria* or *Hirsutella* spp., though documented as nematophagous fungi, may not be considered for the role due to their known disease-causing abilities in plants (Kumar *et al*., 2002; Logrieco *et al*., 2009).

8.3 Need for Regulations to Govern the Use of Nematophagous Fungi

Although biocontrol agents such as fungi are regarded to be safer than chemical control agents when used in crops and fields, they require regulations to govern their use. This particularly applies when exotic organisms are used due to restrictions on importation. Their behaviour may also be altered in the new soil ecosystem. This phenomenon has been used to explain the failure of many nematophagous fungi when used in different soil ecosystems. Many parts of the world have comprehensive procedures in place regulating fungal biocontrol agents. However, in some instances where biopesticide qualities of organisms have been demonstrated, they have sometimes not been developed beyond the experimental stage (David, 2003) partly due to a lack of guidelines and regulations on registration, particularly in developing countries (Cherry, 2006). In developing countries there may be further constraints in the development of potential biopesticide candidates due to poor infrastructure and communications, inadequate extension and training services, financial constraints from funders and the national requirements of individual countries for which manufacturers must comply (Maniania and Lohr, 2003). The markets for which the biopesticides are intended and the ease of use by farmers also need to be taken into account (Maniania and Lohr, 2003). Furthermore, even the availability of biocontrols such as nematophagous fungi products may limit their use, as there may be few companies that produce or trade in these products (Jäkel, 2003). However, it is also recognized that there are constraints in developed regions too, such as the European Union (EU) (Guillon, 2003). These constraints might include poorly drawn-up registration procedures, the specificity of the products which naturally restricts their markets, their mode of action (regulation rather than elimination) and restrictions imposed by certain markets. Ultimately, this may be due to the high cost of production and registration, compared to the potential revenue (Guillon, 2003). In order to facilitate the development of biocontrol agents such as

microbials, regulations are constantly under review with recommendations being updated (e.g. REBECA – Regulation of Biocontrol Agents, in the EU).

Once a potential fungus has been identified, it needs substantial scientific and technical development to determine feasibility for use and to investigate safety issues. Furthermore, as indicated, in each country there are regulations for their use to offset possible safety issues (particularly with exotic fungal organisms) and to ensure quality control and efficacy. On the biopesticides webpage of the UK Government Environmental Protection Agency (http://www.pesticides.gov.uk) the following statement is made 'Before any biopesticide can be used, sold, supplied, advertised or stored it must be approved for use'. This statement is made to demonstrate to all those having interests in biopesticides that their use is subject to regulation and that in a given country they require registration for use on a given crop. Different countries have slightly different approaches but there is a general set of requirements and controls. In many cases the registration requirements may be similar to and based on those for pesticides. Unfortunately, sometimes there are instances of unregistered products being marketed that have not undergone quality control testing for efficacy, consistency and safety (Wozniak, 2003). These may lead to poor success rates that will only serve to alienate farmers from biopesticides such as nematophagous fungi.

Although fungi may be considered as minimal risk biopesticides, it is still necessary to have regulatory procedures for their use. Registration is necessary, as in most cases the modes of action of the nematophagous fungi may not be fully known. New nematicidal fungi are being discovered all the time. Currently, there are more than 200 species of nematophagous fungi described (Tunlid and Ahrén, 2011). Even when the nematicidal abilities of these fungi are understood, their interactions with both human and other animals may not be known in terms of pathogenicity, infectiveness, toxicity, sensitization, allergenicity and carcinogenicity. Some nematicidal fungi may have adverse effects on some plants and soil. For example, *Cochliobolus sativus* has been shown to have nematicidal effects against the juveniles of *Melodogyne* spp. (Mubyana-John and Wright, 2011) while other researchers have documented some strains of *C. sativus* as causal agents for common root rot disease in crops (Kumar *et al*., 2001).

Although legislation and regulations exist in the developed world on the use of biocontrol agents such as nematicidal fungi, this is not always the case in the developing world. Developing countries need to generate their own registration and risk assessment guidelines, which may optionally be based on the standards already available in developed countries and regions like the EU and the USA and also by organizations such as the Interstate Committee for Drought Control in the Sahel (CILSS) (Cherry, 2006). Realizing the need for legislation, some African countries such as Kenya (Maniania and Lohr, 2003) and Ghana (Cherry, 2006) and Asian countries such as Thailand and India have tried to put together regulations to govern the use of biocontrol agents (Pawar, 2001; Grzywacz, 2003; Jäkel, 2003; Rushtapakomchai, 2003). However, these regulations do not necessarily target fungi.

8.3.1 General outline for registration

The application for a product evaluation should be made to a national body responsible for environmental health. In most developing countries it is usually the Ministry of Agriculture. However, in others it could be the Ministry of Environment and Health. In the USA, EU and Canada there is usually a designated body or member state that handles the application.

Registration generally may proceed as follows: the nematophagous fungus needs to be identified and characterized; its biological, physical and technical properties need to be demonstrated and assessed and toxicological and eco-toxicological studies need to be carried out. Therefore, once a particular fungus has been laboratory proven to be nematicidal (preferably with peer-reviewed articles to assess its properties), growth chamber proven and field tested, an application has to be made to legalize its use in the particular country of isolation and testing. Applications for approval must be made using the relevant application

system depending on the country. Before the product can be commercialized, approval has to be granted. Approval can only be given once all the data and/or information on the safety, efficacy and consistency of the fungus are considered to be acceptable. All applications for use of a product must be supported by data. Data must be generated from work carried out to certain standards by appropriately recognized organizations. For instance, in the UK, laboratories must be Good Laboratory Practice (GLP) accredited and field work must be carried out by officially recognized organizations.

Thus in the application, an analysis of the formal data required should be laid out. The data should include a formal dossier. The information below contains the summary of requirements of the Organization for Economic Cooperation and Development (OECD, 2004) (adapted from OECD Guidance for Industry Data Submissions for Microbial Pest Control Products and their Microbial Pest Control Agents (MCPA). Some of it is also derived from an EC directive (Directive 2001/36/EC of 16 May 2001, an annex of EC Directive 94/414) as outlined by Guillon (2003) and is similar in summary to the requirements of the US EPA, which has produced a pesticides registration manual (EPA, 2013) OECD Biocontrol Fungal Guidelines which are mentioned below:

1. The identity of the fungus needs to be established using the best technology available, and also its provenance and biological properties must be reported. Additives to formulations for application must be given. Relevant literature on the organism must be provided. This will require background information that consists of detailed biological properties of the fungal strain and all that is known or documented about the fungi. All the laboratory studies performed should state the analytical methods that were used to assess the fungi.
2. Efficacy and crop safety trials with proof of a consistent level of control and effect.
3. Information regarding how the preparations containing the fungus should be used, dosage and handling procedures should be outlined and the function of the fungi to control nematodes should be demonstrated. The type of situation in which the preparation would be used should also be indicated (e.g. glasshouse, greenhouse, field etc.) and the type of crops it might be registered on. Furthermore, information on the likelihood of resistance developing, a loss of virulence of the fungus and safety procedures for handling, storage, spillage and destruction of the preparation need to be determined.
4. Analytical methods for control after registration and for monitoring purposes must be given with methods for both the analysis of the fungus and any metabolites and residues.
5. Fungal human and animal interactions. Fungal interactions with humans regarding all aspects known and also relevant studies being conducted worldwide.
6. Fungal interactions with other animals apart from humans.
7. Survival aspects of the fungi in the soil after the crop has been removed, any residual effects of the fungi should be given.
8. The fate of the fungi and its general behaviour in the environment. In the event that the fungi is transported and used outside its native climatic region and soil, how well does it survive and would it leave any unwanted residues?
9. The interactions between the fungi and other soil microorganisms such as bacteria and algae.
10. The interactions between the fungi and other soil meso- and macro-organisms such as earthworms and small soil arthropods.
11. Toxicology and exposure data information of the fungi. Fungi have the potential to produce metabolites that are of concern to human health and the environment.

8.3.2 Product information requirements

Generally all that is known about the fungi and its interactions with the rest of the non-target organisms should also be given. This information is necessary for authorization of the preparation as a plant protection product, which includes: identity of the product; technical, physical and chemical properties; information on application methods; packaging,

storage and disposal; efficacy; effects on human health; residues; fate and behaviour in the environment and effects on non-target organisms. The product has to show the efficacy data associated with the fungi use. In cases where the fungi is in a carrier formulation consisting of several fungi, clear understanding of all the fungi and what they are proposed to do (active ingredient and other co-formulants present) should be clearly outlined.

In most cases the nematicidal fungi may be carried in a formulation. A check list for possible impurities in the formulation should be included. The possible side effects and negative interactions that may arise due to the impurities should also be indicated. In the event of possible contamination, the possible consequences on the end-user and the efficiency of the product should be listed.

Interactions between the fungal biocontrol agents and the environment

A clear understanding of the natural background levels of the particular fungal population in the given soil should also have been thoroughly studied. This efficacy could be specific to a country or a particular climatic zone. One of the major obstacles facing the use of introduced alien species for biological control purposes is the difficulty of establishing the efficacy of the organism in the existing soil ecosystem (Carlile *et al*., 2001). This is because of the failure of some nematophagous fungi to adapt to a new environment.

The nematode's ability to destroy the host plant differs with the nature of the crop, with some crops being more susceptible to the infection than others. Crops such as carrots, tomatoes, potatoes and other members of the Solanaceae family are highly susceptible to nematode infection while cereals and other members of Gramineae show some resistance (Bourne and Kerry, 1998). It may be necessary to state if the fungal formulation can be used in all crops or specific crops as the survival of the fungi in the rhizosphere of different crops will differ irrespective of nematode occurrence in their rhizospheres. Bourne and Kerry (1998) showed that plant species differ in their ability to support the growth of the fungus in their rhizosphere; upon application, increase in the fungal application rate resulted in increase in the fungal population in bulk soil at the end of the experiment but they did not consistently increase the amount of fungus in the rhizosphere and rhizoplane. Bourne *et al*. (1996) also showed that an increase in the amount of the fungus applied to soil did not significantly increase the density of the fungus on the roots of aubergine (*Solanum melongena*), whereas on kale (*Brassica oleracea*) and to a lesser extent on bean (*Phaseolus* spp.) root, increase in fungal abundance occurred on both roots and the bulk soil.

Apart from the above, other data may be required in the application. In most cases the nematophagous fungi may be carried in a formulation. However, the active ingredient, i.e. fungal mycelium or spore density, should be accompanied by identification studies that have been carried out and confirmed by the applicant or the designated parties. It should also be necessary to provide the production methods and quality control methods for the active ingredient in the formulation. A check list for possible impurities in the formulation should be included. The possible side effects or interactions that may arise due to the impurities should also be indicated. In the event of possible contamination, the possible consequences on the end-user and the efficiency of the product should also be stated.

Toxicological and eco-toxicological information

Information on fungal × human interactions should clearly indicate if any pathogenicity and infectiveness, is known as this is very important for the safety of farm personnel. Fungi have long been known to have the potential to produce metabolites and properties that may negatively affect humans causing diseases ranging from topical skin infections, acute and chronic toxicity, to systemic diseases. Although it may not be possible to assess the sensitization and allergenicity of the fungal formulations directly, it may be possible to assess this based on the chemical compounds the fungi may produce when grown *in vivo* (Hussein and Brasel, 2001). Knowledge on carcinogenicity of the fungi should be included wherever possible. Knowledge on the

relationship of the fungi to known human dermatophytes is also very crucial and whenever known should be included. These have to be clearly explained in any registration application. Overall, a summary of the fungi's potential to be hazardous to humans with consideration of its pathogenicity, infectivity and pattern of clearance and toxicological effects may be demanded in the EU, Canada and Australia. In order to ascertain this knowledge, studies on acute infectivity, toxicity and pathogenicity have to be performed in the oral, intratracheal or inhalable and intravenous or intraperitoneal routes. If production of exotoxins is not known, then clastogenicity (Ames tests) and gene mutation tests may be required.

Environmental aspects are also supposed to be considered, i.e. fate and behaviour of the fungi in the environment. These should include survival aspects of the fungi in soil and its longevity. The question whether the fungi can only be used where it is indigenous to the area or not should be clearly stipulated as this may have direct implications on the survival of the fungi in relation to the duration of the crop cultivated. It also may have implications on the fungi surviving at minimal levels when the crop is not in place or the nematode population is insignificant. This is important in order to render the nematophagous fungi harmless to other organisms when the preferred substrate (nematodes) is not available. The production of toxins and the potential for multiplication in the environment is normally based on the literature available as this can be overwhelming. Eco-toxicity tests are normally performed on unformulated microorganisms and therefore testing on nematophagous fungal formulations may not be required in some countries unless negative effects on non-target microorganisms are suspected (Strauch *et al*., 2011).

Although nematophagous fungi formulations may be of minimal threat to groundwater systems due to their aerobic nature, they may cause problems if they find their way into polluted surface water, where they could replicate and persist and be a threat to aquatic life. Thus, survival of nematicidal fungi in surface water and its effect on both aquatic fauna and flora is of significance in their use in controlling nematodes. Sometimes procedures for decontamination of water may be required as part of the measures to render the fungi harmless in cases where water pollution occurs.

Interactions between the fungi and non-target organisms in the environment

This requires information on the host range of the fungus. This will include information on the fungal interaction with other microorganisms. This is particularly important for microorganisms that are involved in nutrient cycling. Fungi such as *Trichoderma* have been shown to lyse the cell wall of other microorganisms (Howell *et al*., 2000). Although the nematophagous fungi are used below ground on the plant root systems, the fungi should not have a negative interaction with beneficial insects such as bees which are involved in pollination. Information on the nematophagous fungal–insect interactions is also important as some insects such as ladybird beetles are highly essential in the control of insect pests such as aphids. Consideration for avian toxicity should also be taken into account. In the USA, previously the avian injection test was employed, however, now the inhalation test is being recommended when toxicity to birds is suspected (REBECA, 2007).

The mode of action for the fungi in the control of the nematodes should be fully understood. Where possible the molecular relatedness of the fungi to its close relatives should be shown in order to able to assess possible pathogenicity. Some countries have requested applicants to supply information on the physical and genetic stability of the fungi. It may be necessary to offer a description of extra chromosomal elements involved in the nematicidal activity, pathogenicity or toxicity. Since nematophagous fungi are used below ground, knowledge of the fungal–earthworm interaction is essential due to the role earthworms play in improving soil structure and fertility. Some REBECA experts have proposed waiving of data requirements on the effect of nematophagous fungi on earthworms, as earthworms are well adapted to a broad spectrum of soil-borne pathogens (Strauch *et al*., 2011). However, it may still be necessary to include that information because the mode of action of some nematophagous fungi, such as

A. oligospora and *Arthrobotrys amerospora*, in the control of nematodes include production of protease enzymes (Tunlid and Ahrén, 2011), which could negatively affect earthworms.

Owing to the wide range and diverse nature of information required prior to submitting an application for registration, in some countries such as the USA, EPA may invite the applicant to pre-submission meetings, where the applicant is advised on which studies are necessary, based on the preliminary data and literature available to the applicant. The pre-submission meetings are necessary not just to provide the applicant information needed, but also to decide whether the nematophagous fungi can be given data waivers in some aspects. In Canada, some fungi are regarded as reduced risk pesticides as long as the particular fungi are not documented to be a pathogen. In Australia, at the pre-submission meetings for microbial pesticides, the taxonomy and literature search on possible risks information is used for initial evaluation.

8.3.3 Financial and time aspects of registration

Application fees will differ from country to country and often it can be very expensive. In the UK, the description 'Biopesticide' covers a wide spectrum of potential products, however, application for registration of all microbials, i.e. bacteria, fungi, protozoa, viruses and viroids costs £22,500 (CRD, 2013). However in the EU, this can range from some countries not requiring fees with up to €42,000 in others. Depending on the country, small businesses and governments are exempt from the application fees. Apart from application fees, the large amount of information required in the processes of preparing the application and laboratory work required is also expensive. Therefore, grants for registration of biopesticides are often awarded by some countries.

Once all the documentation has been submitted, the product can be considered for registration. The registration time can also often be a limiting factor. In Canada and in the USA, EPA registers a microbial product in 16 months to 5 years (REBECA, 2007). Subsequently, a re-evaluation of the product would be carried out after 15 years of use. The re-evaluation process is aimed at updating the information available on the product and also at replacing the more risk-prone and outdated fungi handling techniques with modern and lower risk protocols. In Australia, the registration procedure may take 12 months and could take longer depending on the completeness of the information available. In scenarios where the registration processes take longer than anticipated it may disadvantage the farmers. Even for industry, the long waiting periods for registration may often result in delayed onset of returns on the product and also shorter periods of sale under the patent protection period.

Owing to the lengthy procedures in assessing and registering microorganisms some countries such as Italy have introduced the 'Qualified Presumption of Safety' (QPS) concept, which allows the commercialization and use of several products traditionally used in organic farming not registered to be used without individual assessment. A more generic listing and grouping of the microorganism compounds available and in use was introduced in Italy (REBECA, 2007). However, fungi are excluded from this regulation. The main reason is fungi have a complex taxonomy and as such specific knowledge concerning their production of toxic compounds can be complicated.

8.3.4 Limitations of registration

In developed countries the procedures and registration periods may be long (REBECA, 2007). However, for countries such as the USA, provisional national registration may be obtained and efforts are being made to reduce the time and costs associated with registration of biopesticides compared to conventional pesticides (Wozniak, 2003). On the contrary, some countries such as Cuba and India have taken a different approach. Certain categories of microbial pesticides such as indigenous microorganisms that have never been documented as pathogens and are pathogen target-specific have a more straightforward environmental

impact assessment and ecological testing (Diaz, 2003), thus registration periods are reduced tremendously.

Due to the stringent laws on registration of biopesticides, the process may be considered as too severe and an unnecessary impediment to biopesticide development (Blum, 2002). To combat this, India and Thailand have introduced successful initiatives to promote biopesticides based upon indigenous microorganisms (Warburton *et al*., 2002). This was done because the length of processes of biopesticide registration and inappropriate regulation that poses a particular challenge can seriously impede the adoption of new biopesticides, therefore denying farmers access to a potentially viable natural resource. Therefore, these countries have allowed the development of biopesticides to an advanced stage before they can be registered (Grzywacz, 2003). Nematicidal fungi are mostly used in horticultural crops and therefore farmers in countries with less stringent regulations and lengthy procedures are most likely to benefit from newly discovered nematicidal fungi before the ones in countries with stringent regulations and registration protocols. Outside the USA and Canada, biopesticides are typically developed by small to medium enterprises that often lack financial resources and because nematicidal fungi are often niche specific, i.e. they specifically target nematodes, unlike chemical pesticides, the market may be limited. Also, they yield small profit compared to chemical pesticides, thus stringent laws could actually disadvantage their use (Jarvis, 2001).

It is widely acknowledged that the process of developing biopesticides, including biocontrol agents of phytonematodes, is costly (estimated US$1 million for entire product development) (Guillon, 2003), time consuming (the whole process of development from production and formulation, through registration and marketing of the product, to field application can take 7 years) with low returns on investments (Guillon, 2003; Muschler and Roettger, 2003). Therefore, there is a need to increase the availability of biocontrol products to farmers and this can be done by comprehensive extension services with effective technology transfer that requires raising awareness and implementation of the effective use of these products (Evans, 2003). Despite these constraints there is a need and a future for these products. If used properly, they are generally safe and effective and form important contributions to an integrated pest management programme.

8.3.5 Application, training and capacity building

Apart from the input of researchers in determining potential candidates for biopesticides and the response of small- to large-scale industries in product development within the appropriate regulatory framework, another important aspect to their effective use is proper extension services, training of farm personnel and capacity building. Training of extension officers is necessary for them to explain to the farmers the protocols, handling and effective use of the nematophagous fungi. It is essential that the products put into use are properly applied for the most effective outcomes and this is seen as a major obstacle to the use of biopesticides in developing countries where such services may not be available and poor infrastructure imparts limits (Maniania and Lohr, 2003). Locally produced nematophagous fungi may offer a more cost-effective method of nematode control for farmers, provided they are properly produced following the local regulations. It is necessary for the extension workers to be well informed about the product so that they could help guide the farmers on its use.

8.4 Inoculation Methods and Safety Regulations

The problems associated with toxicity, contamination and disposal of properly registered biopesticides appear to be limited in comparison to their chemical counterparts (Chandler *et al*., 2011). However, inoculation methods and safety regulations must be

stipulated for each product, and as with chemical pesticides these must be strictly adhered to for the most effective and safe results. Other advantages in terms of safety are the lack of residues and the low pre-harvest interval achieved (BPIA, 2013).

8.4.1 Safety issues related to handling

The fungal cultures to be used are usually carried in different formulations. However, it is important to state the minimum time the formulations can maintain the culture viable for use, i.e. a verified expiry date should be stated under particular conditions of storage. It has been shown that formulations may survive better at low temperatures, a phenomenon that may prove a challenge in the tropics due to high ambient temperatures and inadequate storage facilities. For instance, the low temperatures may not always be met during transportation of the formulations. Even on arrival at the farms prior to use, farmers may fail to keep the formulations at the stipulated optimum temperature. As such, the expiry dates may also differ with storage conditions. However, in all formulations instructions pertaining to storage of the culture formulations should also be clearly stated. Survival and performance of the fungi may also depend on soil properties such as moisture, pH and organic matter content as these parameters generally influence survival of fungi in soil. In most cases, the fungi are used in the same region and climatic conditions under which they were isolated. However, this is not always the case as some countries may export nematophagous fungi formulations to neighbouring countries. Often this results in failure of the formulation to survive in the new climatic zone and thus the failure to control the nematodes. This has been observed in countries such as Botswana where *P. lilacinus* marketed as 'Pl plus' in South Africa had been imported into Botswana. In many cases farmers reported the formulation's failure to control nematodes (Mubyana, 2002). Closer observations indicated failure of the fungi to survive in the rhizosphere. Therefore, survival of the fungi in the rhizosphere is not just controlled by the crop and rhizosphere effects but also by climatic conditions.

Among other safety aspects to be considered is spore toxicity. In situations where the formulations are available in powder form, during its application it could be inhaled by farm personnel and therefore could result in spore toxicity or allergy reactions to the farm personnel. Therefore, precautionary measures accompanying the formulation should state on how the dust formulation should be used with minimum spore spread risk. In the preparation of the formulations aseptic techniques should be employed as much as possible to ensure production of a non-contaminated formulation. The formulation measures should also state if any aseptic techniques should be employed when working with the formulation to ensure maximum success.

8.5 Requirements for Registration of Fungal Products

For registration, the fungal products should carry the following information mentioned below.

1. Genus, species and strain number.
2. Brief summary of the product describing the fungus and all that is known about its interaction with the environment.
3. Description of the active ingredient in the formulation.
4. Methods of application.
5. Risk assessment to human health.
6. Risk assessment to the environment.
7. Registration information giving the trade name and place of registration.
8. Other additional information, including the contact information of the distributor.

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Part III

Nematophagous Bacteria

9 Nematophagous Bacteria as Biocontrol Agents of Phytonematodes

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9.1 Introduction

Plant-parasitic nematodes (PPN) are a severe constraint to agricultural production worldwide, in turn impacting international trade, social and economic development (Perry and Maurice, 2013). A great deal of research has been done on phytonematodes and their hosts including many horticultural and field crops in tropical, subtropical (e.g. Luc *et al*., 2005), temperate (e.g. Evans *et al*., 1993) and other regions (e.g. Perry and Maurice, 2013). The nematodes can damage their host plants directly, act as vectors of viruses, or form disease complexes with other pathogens. In addition, nematode penetration of infected plants may facilitate subsequent infestation by secondary pathogens such as fungi and bacteria (Powell, 1971). Yet, due to their often subterranean habit and microscopic size, phytonematodes usually remain invisible to the naked eye (Ngangbam and Devi, 2012), which complicates their control. Monetary estimates of global annual yield losses caused by the nematodes demonstrated staggering figures whether in the past (e.g. Sasser and Freckman, 1987; Handoo, 1998; Hodda, 2004) or recently (see Abd-Elgawad and Askary, Chapter 1, this volume).

Therefore, the development of various PPN management methods is a formidable challenge to meet human beings' ambition for the better. Phytonematode management can be defined as some practice(s) aimed at maintaining the populations of various phytonematodes below the threshold level. Consequently, economic losses in host plants are avoided (Usman and Siddiqui, 2013). Because most PPN spend their lives either in the soil or within plant roots, direct contact/efficacy of a chemical nematicide on nematodes is difficult. Moreover, the outer surface of nematodes is a good general protectant. Delivery of a toxic compound by nematode mouth is unlikely. Consequently, synthetic soil nematicides are usually broad-spectrum toxicants with high volatility and spreading (Chitwood, 2003). It is a dilemma that because of such properties, good for nematode control, these nematicides have, on the contrary, a negative impact on human beings and the environment. Therefore, several such chemicals have been banned/withdrawn from the market due to their serious threats to natural biological control processes, wildlife, groundwater contamination, resource depletion and human health and safety. Furthermore, the resulting record of environmental pollution or human health hazards has

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resulted in the broad banning/cancellation of several agronomically valuable chemical nematicides (e.g. ethylene dibromide and dibromochloropropane). For example, in the 1970s, research on the fumigant nematicide 1,2-dibromo-3-chloropropane (DBCP) proved its health hazards. The metabolic pathways of DBCP could explain the toxic nature of the fumigant as a carcinogen, a male chemosterilant and as an agent causing kidney damage (Jones *et al*., 1979). More recently, the decisive fumigant nematicide, methyl bromide, has been banned because of worries about atmospheric ozone exhaustion as well. In addition to their health hazards, many chemical pesticides are expensive or have been withdrawn from use, which has led to increased interest in organic methods for crop and pest management for many crop production systems. In other words, growing dissatisfaction with such chemicals required environmentally friendly pathogen control approaches. These approaches included, for example, the use of grafted vegetable seedlings, especially with watermelon, melon, cucumber, tomato and aubergine (e.g. Abd-Elgawad *et al*., 1994; Lee and Oda, 2003). Yet, development of improved grafting technology and horticultural practices are desperately needed to offset the high cost of a grafted vegetable seedling (Oka *et al*., 2004). Crop rotation, in spite of its effectiveness against the nematodes, also has important limitations. The amount of damage inflicted on a crop depends on the level of resistance in the crops used in the rotation and on the number of years of planting these crops between susceptible crops. Another disadvantage is the low commercial value of some non-host or resistant crops (Sasser, 1989) as well as development of nematode-resistant pathotypes (see Abd-Elgawad and Askary, Chapter 1, this volume). Moreover, some farmers have inadequate land to rotate crops, especially in developing countries.

In fact, the most promising approach to nematode management will combine several tools/devices and tactics, such as the use of cover crops, crop sequences, pre-plant solarization of soil, least-toxic nematicidal material, organic matter/amendments, biological control agents and nematode-resistant plant varieties. Such approaches are more effective for phytonematode-suppressive soils with beneficial populations of microorganisms. Compost or manure additions usually decrease nematode pest populations and accompanying damage to crops (Chen *et al*., 2000). Such additions improve soil quality, enhance plant resistance, emission of nemato-toxins and/or enhanced beneficial populations of fungi/bacteria and other nematode antagonistic agents (Bulluck *et al*., 2002; Usman and Siddiqui, 2013). Coping with the emerging new wave in agriculture, which sees off applications of hazardous chemical nematicides, replacing them with safe biological control agents, a great deal of effort has gone toward nematode and management research on various nematode-susceptible crops. The advantages of these biocontrol agents, such as beneficial bacteria, are acceptable from agricultural specialists and non-specialists because these agents save the environment from polluted air, soil, animals and plants as well as protecting human beings from numerous chronic, acute and irrevocable diseases. Biological control of agricultural pests, whether conducted as classical, augmentation or conservation biological control, necessarily plays a pivotal role. When considering the costs and benefits of various management paradigms, it is noteworthy that increased chemical inputs do not necessarily result in increased output per unit area. For example, in the Mediterranean Basin, Spanish and Italian citrus growers use four-fold and 15-fold greater quantities of pesticides than do Greek growers, but obtain similar citrus production per hectare (Gutiérrez *et al*., 2005). Nevertheless, pest management is of frequent and critical concern in all agricultural endeavours, and the development of sustainable pest management systems requires a fundamental understanding of how populations of pests and their natural enemies behave in specific crop habitats (Campos-Herrera *et al*., 2009). Several developments have recently advanced the field for the biological control of phytonematodes (Stirling, 2014). In this respect, the enhanced activity of microbes within the amended soil may result in the liberation of a wide range of chemically different substances, which may have different levels of toxicity to phytonematodes. For example, Orion *et al*. (1980) indicated that higher concentration

of ammonia released during the decomposition of organic additives/amendments could inhibit the complete development of syncytium which is necessary for the nematode life cycle, since syncytium is the food supplier for the root-knot nematode. Such a medium for growing crops is called a nematode-suppressive soil. Therefore, research and reports on such microbial agents that work against phytonematodes and do not have a detrimental impact on human beings and the environment are becoming increasingly important.

In numerous nematode-suppressive soils as well as in monoculture systems, nematophagous bacteria thrive under some crops and consequentially demonstrate positive effects in suppressing economically important phytonematode genera/species (Stirling, 2014). Advances in the last decades have produced a number of nematophagous bacteria-based products, containing live microorganisms or their metabolites that are already marketed. Some of the well-accepted commercial products contain the bacteria *Bacillus firmus* and *Pasteuria penetrans* (Lamovšek *et al*., 2013). Such products are currently considered prime candidates as biocontrol agents against phytonematodes, with others in the pipeline. For example, bacteria of the genus *Pasteuria* could work as promising biocontrol agents for PPN. Isolates of *P. penetrans* have been shown to reduce root-knot nematode (RKN) damage below economic threshold levels and to enhance yield parameters of the host crops (e.g. Chen and Dickson, 1998; Chaudhary and Kaul, 2011; Mukhtar *et al*., 2013). *Pasteuria* endospores (Fig. 9.1) have additional merits due to their deep-rooted resistance to heat, drying and mechanical shearing. Each *Pasteuria* species is highly host specific and has an excellent safety profile. Until a few years ago, *Pasteuria* has never been produced commercially. A seminal step in nematophagous bacteriology in terms of *Pasteuria* occurred with the development of efficient mass-production technologies. In addition, endospores of *Pasteuria* could be isolated and produced from the sting nematode, *Belonolaimus* spp., and growth of *Pasteuria* from cyst, lance and lesion nematodes in tissue culture plates has been demonstrated. Products controlling this group of nematodes may strengthen other tactics for pest management in turf, vegetables and field crops (http://www.ipmcenters.org/ipmsymposiumv/posters/022.pdf). Yet, it is well known that such microorganisms are generally slower, less successful and less firm in achieving conclusive results than synthetic chemicals in pest control (Kerry, 1990; http://www.fao.org/docrep/v9978e/v9978e0b.htm). On the other hand, improvements in their efficacy might be gained, and therefore we will consider herein the key factors for more effective biological control agents for PPN in terms of nematophagous bacteria. Admittedly, their successful application will depend on our comprehensive knowledge of their behaviour and interaction with different biotic and abiotic factors in specific habitats. The modes of action of nematophagous bacteria may be through: parasitizing; producing toxins, antibiotics, or enzymes; competing for nutrients; inducing systemic resistance of plants; and promoting plant health (see Tian *et al*., 2007; Rocha and de Souza, Chapter 10, this volume). We intend herein to present important aspects of nematophagous bacteria and their impact on PPN such as fundamentals for their development as biocontrol agents of phytonematodes, their host range/specificity and epidemiology. These fundamentals are necessary for an authentic appraisal of their impact as biological control agents against phytonematodes and for their further improvement and following the distribution and persistence of applied bacteria, as well as for the evolution of optimum strategies for PPN control.

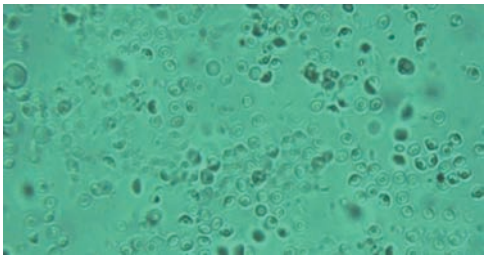

Fig. 9.1. Oil immersion view of *Pasteuria penetrans* spores isolated from infected *Meloidogyne incognita* female (from Chaudhary and Kaul, 2011, with permission).

In this chapter we have tried to focus on live bacterial action towards the phytonematodes

and consequently their economically important host plants. Recent advances used in this field as a background to support future research will be considered. More details are found about their virulence mechanisms (see Rocha and Souza, Chapter 10, this volume), survival biology (see Alves and de Souza, Chapter 11, this volume), field application and commercialization (see Abd-Elgawad and Vagelas, Chapter 12, this volume) and novel bacteria species in nematode biocontrol (see Vagelas, Chapter 13, this volume).

9.2 Groups of Nematophagous Bacteria

Here, we group nematophagous bacteria based on their modes of action; the nematophagous bacteria can be broadly grouped into parasitic bacteria and non-parasitic rhizobacteria (Siddiqui and Mahmood, 1999); the latter group was further split to contain additional categories. Herein we will discuss the six groups of nematophagous bacteria as categorized by Tian *et al*. (2007).

9.2.1 Obligate parasitic bacteria – *Pasteuria*

Background, members and life cycle

The genus *Pasteuria* Metchnikoff, 1888 was first assigned within parasites of water fleas, *Daphnia magna* Straus (a bacterium). Metchnikoff (1888) erected the bacterium *Pasteuria ramosa* but his efforts to culture the bacterium were not fruitful. Cobb (1906) had the first statement that 'an organism resembling *Pasteuria* sp. infecting a nematode, *Dorylaimus bulbiferus*'. He mistakenly thought that the spores inside *D. bulbiferus* were 'perhaps monads' of a parasitic sporozoan. Such a description held true till the 1970s, when this parasite of nematodes was closely examined with electron microscopy and found that it was surely related to bacteria and not protozoa. Therefore, he called it *Bacillus penetrans* (Mankau 1975a,b). Sayre and Starr (1985) threw more light on *B. penetrans*, which resembled the actinomycete *P. ramosa*, and changed its scientific name to *Pasteuria penetrans* (Chen and Dickson, 1998). The genus *Pasteuria* as erected by Metchnikoff has been maintained (Starr *et al*., 1983; Judicial Commission of the International Committee on Systematic Bacteriology, 1986). *Pasteuria* is gram-positive, 'mycelial' and endospore-forming (Fig. 9.1). Its species are endoparasitic prokaryotes that have global spreading in soil and water (Preston *et al*., 2003). Examples of bacteria that can form endospores include also *Bacillus* and *Clostridium*. The name 'endospore' is suggestive of a spore or seed-like form (endo means within), but it is not a true spore (i.e. not an offspring). An endospore is a dormant, tough and non-reproductive structure produced by certain bacteria. An endospore usually consists of the bacterium's DNA and part of its cytoplasm, surrounded by a very tough outer coating. However, *P. penetrans* spores have structures that contact the cuticle of second-stage juveniles (J_2) of *Meloidogyne incognita*. The spores usually adhere to every part of the J_2 cuticle (Fig. 9.2). Spores that measure about 2.7 µm in diameter, adhering to the surface of root-knot juveniles, are considered mature. The exosporium membrane does not exist. The spore has two parts: a central component, 0.9 µm in diameter, and a peripheral surrounding matrix, 1.8 µm in width (El-Saedy and Mokbel, 2007). Endospore formation is usually triggered by a lack of nutrients and so enables the bacterium to tolerate harsh conditions. Endospores enable bacteria

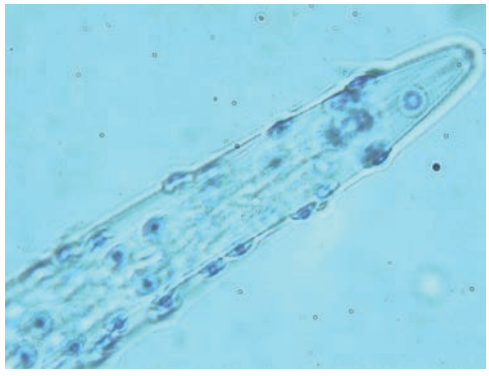

Fig. 9.2. The oil immersion view of *Pasteuria penetrans* spores attachment on and in the body of *Meloidogyne incognita* second-stage juvenile (from Chaudhary and Kaul, 2011, with permission).

to lie dormant for extended periods, therein providing another advantage in the use of these bacteria as a biocontrol agent. Moreover, they are resistant to ultraviolet radiation, desiccation, temperature extremes (high and low) and chemical disinfectants. Common antibacterial agents that work by destroying vegetative cell walls do not affect endospores. Endospores are commonly found in soil and water, where they may survive for long periods of time. When the environment becomes more favourable, the endospore can reactivate itself to the vegetative state.

The genus *Pasteuria* has, so far, four recognized species: *P. ramosa* (Metchnikoff, 1888) has been found in water fleas, *P. penetrans* (Sayre and Starr, 1985) primarily parasitizes RKNs (*Meloidogyne* spp.), *Pasteuria thornei* (Starr and Sayre, 1988) parasitizes root-lesion nematodes (*Pratylenchus* spp.) and *Pasteuria nishizawae* (Sayre *et al*., 1991; emended by Noel *et al*., 2005) occurs on both genera of the cyst nematodes *Heterodera* and *Globodera* (Atibalentja *et al*., 2000); and two '*Candidatus*' taxa, '*Candidatus* Pasteuria usgae' and '*Candidatus* Pasteuria aldrichii'. They are parasites of the sting nematode (*Belonolaimus longicaudatus*) (Giblin-Davis *et al*., 2001, 2003) and the bacterivorous nematode *Bursilla* (Giblin-Davis *et al*., 2011), respectively. Being obligate endoparasites, *Pasteuria* could not be isolated as definitive type strain and consequently '*Candidatus*' is suggested (Giblin-Davis *et al*., 2003, 2011). Most current *Pasteuria* research is on phytonematode-parasitic *Pasteuria* species. This bias is probably due to a rooted interest in materializing these bacteria as biocontrol agents to target a specific group of major nematode pests. So, it is not a proof that any definite affinity of the afore-mentioned bacteria for a fixed trophic group of these roundworms exists (Sturhan *et al*., 2005; Giblin-Davis *et al*., 2011). The search for other new species of *Pasteuria* that can provide effective control of a persistent phytonematode pest – and be commercially marketed as such – is on and, therefore, we anticipate additional novel promising species in the future, especially in parallel to growing dissatisfaction with chemical nematicides.

Pasteuria taxonomy depends mainly on structural and pathological traits, including the measurements (size and shape) of sporangia and endospores, and ultra-structures, life cycles and host ranges (Tian *et al*., 2007). Analysis of a portion of the 16S rRNA gene demonstrated *Pasteuria* as a stable member of the *Clostridium–Bacillus–Streptococcus* group in Eubacteria (Anderson *et al*., 1999). More recently, molecular bio-analyses are being used for accurate characterization of this genus. *P. penetrans* genome was sequenced, amino acid-level analysis was done and consequently *P. penetrans* was considered as ancestral to *Bacillus* spp. (Charles *et al*., 2005). The results suggested that *P. penetrans* might have developed from a primary symbiotic bacteria associate of nematodes, presumably at the time of RKN evolution to a high form of plant parasite. Culturing of *Pasteuria* has been achieved only recently due to the obligate parasitic nature of members of this genus. It is only attained through proprietary means without adequate terms for the promotion and continuous maintenance of a standard collection of the bacteria. An ideal method that is based mostly upon the mature endospores' ultrastructure and on host-range investigations to designate taxa was used (Giblin-Davis *et al*., 2011). However, recent developments have resulted in a molecular phylogenetic approach to identify/designate different taxa of *Pasteuria* (Schmidt *et al*., 2010; Luc *et al*., 2011b).

Pasteuria penetrans completes its life cycle within a short time (18–20 days) on different nematodes it is parasitizing, but the time to complete this cycle also depends on other factors such as temperature (Stirling, 1981; Siddiqui and Mahmood, 1999). Five spores per nematode were required for infection; females, males and juveniles of *Meloidogyne javanica* became infected with spores (Stirling, 1984). Generally, the life cycle of *Pasteuria*, which occurs on phytonematodes, begins when the endospores adhere to the nematode cuticle. This first interaction between *Pasteuria* and the nematodes occurs when the worms move through soil infested with endospores; for example *P. penetrans* attachment to J_2 of RKNs (Fig. 9.2). Such attached endospores germinate within 4 to 10 days on *Meloidogyne* spp. J_2, which can move into the roots (Chen and Dickson, 1998). A bacterial tube emerges and penetrates the outer layer (body wall) of the nematode. The process of penetration

is facilitated by enzymes (Mankau, 1975a,b). Having entered the pseudocoelom of PPN, the germ tube develops proliferation of septate mycelium that is branched bilaterally. Nascent colonies are established. The colony then fragments, and the terminal cells of each fragment always develop and undergo sporogenesis (the process of spore formation); the factors responsible for such development have not been studied, though the process of spore formation is essential for the life cycle. In this cycle, doublets and quartets of developing sporangia predominate in the nematode-body cavity and then they separate into individual sporangia. Finally, the mature endospores are transferred to soil when the infected root, having the bacteria in the form of parasitized RKN females, decomposes. Sporogenesis has been divided into seven stages (Chen and Dickson, 1998) where the bilaterally branched vegetative mycelium of *P. penetrans* has been considered as the initial stage 0, which is comparable to the *Bacillus* spp. vegetative cells.

Pasteuria *in the biocontrol of phytonematodes*

Many isolates of *Pasteuria* occur worldwide and researchers have been studying their efficacy on phytonematodes and their host plants in many countries (e.g. Starr and Sayre, 1988; Anderson *et al*., 1999; Jonathan *et al*., 2000; Giblin-Davis *et al*., 2003; El-Saedy and Mokbel, 2007; Tzortzakakis, 2008; Luc, 2009; Chaudhary and Kaul, 2010; Senol and Handoo, 2012; Mukhtar *et al*., 2013; Stirling, 2014). These studies aim at offering efficacious and cost-effective *Pasteuria* as safe and environmentally-friendly nematicides. Members of *Pasteuria* are listed to parasitize 323 nematode species within 116 genera, including both PPN and free-living nematodes (Chen and Dickson, 1998). Most economically important PPN have been documented in this list (Bird *et al*., 2003). Admittedly, no one was able to grow *Pasteuria in vitro* until Dr John Gerber demonstrated that the complete life cycle of *Pasteuria* could be done *in vitro* thus opening the door for marketing products with *Pasteuria* as the active ingredient (see Abd-Elgawad and Vagelas, Chapter 12, this volume). Yet, improvement in the quality, mass production, formulation, packaging and application of promising *Pasteuria* species/isolates should continue as these are considered the milestones to enhance efficacy and consumer acceptance of *Pasteuria* products. This is especially important because biocontrol agents are generally more inconsistent but possible improvement is attainable.

Greenhouse experiments indicated that *in-vitro* produced (IVP) endospore movement into the turf zone is not hindered by thatch and that many periods of irrigation can move endospores below the turf-grass root zone, possibly reducing effectiveness. In addition, placement of endospores within the root profile (0–10 cm soil depth) with one application of 0.6 cm of irrigation suggested that endospore applications can be achieved relatively easy. These trials have supplied further information; however, experiments conducted in a controlled environment do not always correspond to field observations (Luc, 2009). In 2010, a turf-grass bionematicide containing *in vitro*-produced *Pasteuria* sp. for management of *B. longicaudatus* was launched under the trade name Econem. Greenhouse pot studies and field trials on golf course fairways and tee boxes evaluated Econem at varied rates and application frequencies. Trials on putting greens compared efficacy of three applications of Econem at 98 kg/ha to untreated controls and 1,3-dichloropropene at 53 kg a.i./ha. Further putting-green trials evaluated the ability of three applications of Econem at 98 kg/ha to prevent resurgence of population densities of *B. longicaudatus* following treatment with 1,3-dichloropropene at 53 kg a.i./ha. None of the Econem treatments in pot studies were effective at reducing *B. longicaudatus* numbers ($P \leq 0.05$). Econem was associated with reduction in population densities of *B. longicaudatus* ($P \leq 0.1$) on only a single sampling date in one of the eight field trials and did not improve turf health in any of the trials ($P > 0.1$). These results did not indicate that Econem is an effective treatment for management of *B. longicaudatus* on golf course turf (Crow *et al*., 2011). In another trial, ten treatments to evaluate movement of *in vitro*-produced *Pasteuria* sp. endospores into a simulated putting-green

profile in lysimeters included five watering levels: 0.6 cm of water with a wetting agent, 0.6 cm of water without a wetting agent, 2.5 cm of water without a wetting agent, 7.6 cm of water without a wetting agent, 15.2 cm of water without a wetting agent, and two thatch levels: with or without thatch. The endospores were applied as a drench at 1,990,000 endospores/cm^2 of soil surface. A bioassay using *B. longicaudatus* was conducted to determine relative endospore attachment at four depths following the water treatments. Application of 15.2 cm of irrigation reduced percentage endospore attachment by 62% and 39% at soil depths 0 to 2.5 and 2.5 to 10 cm, respectively, and increased percentage endospore attachment by 95% and 2297% at soil depths 10 to 20 and 20 to 30 cm, respectively, compared with 0.6 cm of irrigation (Luc *et al*., 2011a). Moreover, Luc *et al*. (2011b) evaluated the isolate specificity among *in vitro*-produced *Pasteuria* sp. endospores to suppress different *B. longicaudatus* populations. *Pasteuria* sp. were collected from infected *B. longicaudatus* in five different geographic areas, cultured *in vitro*, and tested for their ability, as five distinct isolates, to infect, reproduce on and control two genetically and geographically diverse isolates of *B. longicaudatus* under controlled conditions. The isolates were so similar that no differences ($P \geq 0.05$) were apparent among the *in vitro*-produced *Pasteuria* sp. isolates. All isolates decreased numbers of *B. longicaudatus* nearly 70% compared to the control (pots without endospores). However, these findings differ from previous research conducted with *in vivo*-produced endospores (Davies *et al*., 1994). The latter research demonstrated cuticle heterogeneity in respect to *in vivo* endospore attachment between populations of *Meloidogyne*. Furthermore, populations of RKNs that appeared to be comparatively homogeneous from studies using DNA, exhibited considerable level of variation in the binding of *in vivo Pasteuria* spores. This would suggest the ability of nematodes to develop levels of cuticle heterogeneity faster than phylogenetic analysis can observe. However, phylogenetic analysis of the ITS1, 5.8S and ITS2 regions reported by Luc *et al*. (2011b) indicated diversity between the two *B. longicaudatus* populations designated as WP and SUN populations, but no differences in spore attachment were observed. So, Luc *et al*. (2011b) concluded that the ITS1, 5.8S and ITS2 regions may be better suited for distinguishing between species or populations within a species (Cherry *et al*., 1997) than previously investigated DNA regions, but these differences do not address *Pasteuria* sp.-attachment ability. Bekal *et al*. (2001) showed that soil naturally infested with spores of *Pasteuria* strain S-1 had different levels of percentage endospore attachment (51% to 96%) for geographically different populations of *B. longicaudatus*. Differences in percentage endospore attachment were also observed among three Florida populations (Bekal *et al*., 2001). Therefore, the lack of differences in percentage endospore attachment for the above-mentioned two genetically and geographically distinct *B. longicaudatus* populations (Luc *et al*., 2011b) was not expected, suggesting that a closer look at the effects of the *in vitro* fermentation process on the resulting progeny may be warranted. Schmidt *et al*. (2010) reported that some different *Pasteuria* sp. constitute only biotypes (within the same species) based on phylogenetic analysis of *Rotylenchulus reniformis*, though they showed different host specificities. *Pasteuria* and its species parasitize major PPN. Their practical life cycle points out host preference/specificity but this point needs further research. In order to clarify how the endospore starts to attach, Spiegel *et al*. (1996) conceived a model where they found that binding of lectins to nematode juvenile surface reduced the spore attachment. Eventually, Luc (2009) concluded that biopesticides using IVP may be an important component of integrated pest management (IPM) for *B. longicaudatus* on turf grasses but need further improvements.

Chaudhary and Kaul (2010) demonstrated that time of *P. penetrans* application to soil had profound effect on the magnitude of the phytonematode control by the bacterium and consequently on crop yield associated with the nematodes. The simultaneous application of *P. penetrans*-infested soil with *M. incognita* J_2 was found to be the most favoured for fresh and dry biomass of the chilli crop. The simultaneous application of *P. penetrans* with phytonematode addition caused intense decrease in the final population of *M. incognita*.

This treatment yielded 45–53% improvement in the various biomass parameters of the crop and 71% reduction in final *M. incognita* population. Before that, the numbers of attached spores of three *P. penetrans* isolates did not discriminate virulent from avirulent *M. incognita* and *M. javanica* populations from Greece, within each species; virulent nematodes are able to reproduce, whereas avirulent nematodes are unable to reproduce on a host plant that possesses one or more resistance genes. So, attachment rates probably reflect the specificity of *P. penetrans* to different *Meloidogyne* populations within a species but not to different species, within a definite region. These nematode populations differed in their ability to reproduce on tomatoes with the *Mi* gene, responsible for RKN resistance (Tzortzakakis, 2008).

9.2.2 Opportunistic parasitic bacteria

As early as 1946, Dollfus was able to investigate and document bacteria within nematodes (Jatala, 1986). *Pseudomonas denitrificans* also parasitized populations of *Xiphinema americanum* (Adams and Eichenmuller, 1963). Admittedly, many bacteria are frequently found on the PPN cuticle. Nevertheless, researchers are unable to know whether we can benefit from these bacteria as parasites or whether they are just saprophytes (e.g. Jatala, 1986). Based on many observations and tests, these groups of bacteria can parasitize their available nutrient resources and therefore usually live as saprophytes, targeting PPN as one of many possible nutrient resources. Such bacteria are, however, also able to penetrate the PPN cuticle and put to death a nematode host which the bacteria may encounter. They may be regarded as opportunistic parasitic bacteria here, and were previously exemplified by *Brevibacillus laterosporus* strain G4 and *Bacillus* sp. B16 (Tian *et al*., 2007). *B. laterosporus* has an outstanding profile of pathogenic activities. So far, several authors (e.g. Oliveira *et al*., 2004; Huang *et al*., 2005) have recorded some phytonematodes such as *Heterodera glycines*, and *Bursaphelenchus xylophilus* and the saprophytic nematode (*Panagrellus redivivus*) that could be controlled by numerous isolates of *B. laterosporus*. *B. laterosporus* also parasitizes *Trichostrongylus colubriformis* and *B. laterosporus* strain G4 parasitizes two nematode species *P. redivivus* and *B. xylophilus* (Huang *et al*., 2005). Having attached to the epidermis of the host body, *B. laterosporus* can reproduce quickly on PPN cuticle. Further growth of the bacterium usually results in a circular hole on the PPN cuticle and related tissue. Eventually, bacteria break into inner nematode tissues and feed on them (Huang *et al*., 2005). Throughout the infection course, the degradation of all the PPN cuticle compounds demonstrates the participation of hydrolytic enzymes (Cox *et al*., 1981; Huang *et al*., 2005). Histopathological and molecular studies have proved that most pathogenic performance could be caused by an extracellular alkaline serine protease, known as BLG4 (Huang *et al*., 2005; Tian *et al*., 2006). The most compelling proof which supports the role of protease as the effective factor was demonstrated by studying protease-deficient mutants (Tian *et al*., 2006). The BLG4-deficient strain BLG4-6 was only 43% as effective as the wild-type strain in killing nematodes, but had only 22% as much cuticle degrading activity. Such obtained data may point out that BLG4 is not the only virulence operator that exerts nematicidal action, and that other agents, e.g. other extracellular enzymes or toxins, are probably engaged (Tian *et al*., 2007). In fact, *B. laterosporus* can act as a biocontrol agent of PPN after a nematicidal component from spores of certain strains was found to control egg viability and growth of roundworms. Bone and Singer (1991) characterized the isolated toxin using HPLC. It was a heat stable, low molecular weight protein of approximately 2900 Da, with UV absorbance at 205, 220 and 268 nm.

Additional proteases of the bacteria were proved to participate in the course of infection against PPN. Among them, the serine protease genes from nematode-feeding bacteria extracted from a different location in Yunnan (*B. laterosporus* strain G4, *Bacillus* sp. B16, *Bacillus* sp. RH219 and other *Bacillus* strains) have been detected and compared (Niu *et al*., 2005; Tian *et al*., 2006). The sequences of amino acids related to these bacterial cuticle-degrading proteases demonstrated enhanced sequence identity (97–99%). The coherence of such pathogenic proteases by different nematode-feeding

bacterial strains suggests that these enzymes are greatly conserved in the nematophagous bacteria. Moreover, another neutral enzyme (designated protease Bae 16) of 40 kDa, toxic to nematodes, was purified to homogeneity from the strain *Bacillus nematocida*. The activity was tested against two nematode species. This purified enzyme could destroy cuticle of *P. redivivus* and *B. xylophilus* and its hydrolytic substrates included gelatin and collagen. The gene encoding Bae 16 was cloned and expressed in *Escherichia coli*, confirming its nematicidal activity (Niu *et al*., 2006).

Most investigations and reports on this category of parasitic bacteria are aimed at grasping pathogenesis utilizing saprophytic nematodes as targets. Such targets are easier to use for identifying new pathogenic agents/factors, and to increase knowledge about how infections proceed in PPN. It would be of much interest to illustrate the mechanism responsible for the switch from saprotrophy to parasitism so that we can formulate and develop effective commercial agents from this category for PPN control (Tian *et al*., 2007).

9.2.3 Rhizobacteria

All the bacteria that usually inhabit the region of a plant root may be called rhizobacteria, but due to specific properties in terms of difference in mechanism of action the other bacterial groups, mentioned in the above categorization, were separated from this group. Rhizobacteria have long been considered for PPN control (Sikora, 1992). Their importance varies according to their pathogenicity to phytonematodes and also to humans. For example, a suspension of *Serratia marcescens* reduced *M. incognita* development and reproduction on two tomato cvs, Super Strain B and Alisa, and increased plant growth parameters (Abd-Elgawad and Kabeil, 2010). However, the strain used must be checked for human safety since *Serratia* infection is generally responsible for some human diseases (Castelli *et al.*, 2008). Aerobic endospore-forming bacteria, mostly *Bacillus* spp. and *Pseudomonas* spp., are among the prevailing populations which inhabit the rhizosphere and are able to antagonize PPN (Tian *et al*., 2007). We will give recent examples of numerous rhizobacterial species, strains and/or isolates that can suppress phytonematodes and promote plant growth and/or yield. Admittedly, some rhizobacterial species are pathogens of nematodes (e.g. Li *et al*., 2005; Aballay *et al*., 2011; Ribeiro *et al*., 2012). *Bacillus subtilis* is very frequently found in soil and has therefore received numerous studies. Abd-Elgawad and Mohamed (2006) speculated that the initial population of *B. subtilis* may have come to an arable land from a trace carried to the soil by contaminated air and then could thrive on the ground on the cuticle of ascaris (*Ascaris lumbricoides*). They reported that many *B. subtilis* bacteria existed in ascaris cuticle-treated pots (138 bacterial cells/g of treated soil) with respect to the control (28 bacterial cells/g); it is likely that ascaris cuticle could be used for culturing this bacterium, which apparently could control *M. incognita* (Abd-Elgawad and Mohamed, 2006). However, further evidence suggests that *B. subtilis* is a normal gut commensal in humans. Hong *et al*. (2009) compared the density of spores found in soil (~10^6 spores/g) to that found in human faeces (~10^4 spores/g). The number of spores found in the human gut is too high to be attributed solely to consumption through food contamination. Soil simply serves as a reservoir, suggesting that *B. subtilis* inhabits the gut and should be considered as a normal gut commensal. A number of studies have also recovered *Bacillus* species in mammals as reviewed by Hong *et al*. (2005). On the other hand, a number of studies recorded direct antagonism by other *Bacillus* spp. towards PPN species related to very economically important nematode genera, e.g. *Meloidogyne*, *Heterodera* and *Rotylenchulus* (Tian *et al*., 2007). Rhizosphere *Pseudomonas* strains exhibit diverse pathogenic mechanisms upon interaction with nematodes. Other rhizobacteria deserve more studies since they demonstrate potential to control PPN. Tian *et al*. (2007) stated many genera that may include one or more of such rhizospheric bacteria. Among other mode of actions (see Rocha and de Souza, Chapter 10, this volume), metabolic by-products, enzymes and toxins are used by most rhizobacteria against PPN. Their efficacy is materialized in the form of suppressing nematode reproduction, egg viability and

juvenile infection/activity, as well as direct killing of PPN. *Pseudomonas fluorescens* was used to control cyst nematode juveniles by producing numerous secondary metabolites, e.g. 2,4-diacetylphloroglucinol (Cronin *et al*., 1997; Siddiqui and Shaukat, 2003). *Corynebacterium paurometabolu* reduced nematode egg hatching by producing hydrogen sulfide and chitinase (Mena and Pimentel, 2002). Other rhizobacteria reduce harmful organisms at the same time as creating suitable conditions for enhancing growth parameters of plants. They can manufacture antibiotics or hydrogen cyanide (Zuckerman and Jasson, 1984). Rhizobacteria-mediated induced systemic resistance (ISR) in plants has been studied extensively and proved to work against PPN (e.g. Van Loon *et al*., 1998; Ramamoorthy *et al*., 2001). *In vitro* study on some defence enzymes induced by *P. fluorescens* isolate Pf1 against *M. graminicola* infection in rice demonstrated that such enzyme levels were higher in the bacterized grown rice. The isoform profile analysis indicated unique enzymatic isoforms induced in plants treated with *P. fluorescens*. These outcomes (Anita and Samiyappan, 2012) suggest timely and enhanced bulk of phenols and defence enzymes in the plant tissue causing an important reduction in *M. graminicola* infection. Similarly, Abd-Elgawad and Kabeil (2012) proved that *S. marcescens* may be used to prime tomato plants for RKN resistance. Plant growth-promoting rhizobacteria (PGPR) can bring about ISR by strengthening the physical and mechanical vigour of the cell wall through cell-wall thickening/reforming, deposition of newly formed callose, and aggregation of phenolic compounds. They also raise the physiological and biochemical capability of the host plant to promote the synthesis of defence chemicals against the attacking pathogen (e.g. by the piling up of pathogenesis-related proteins, enhanced chitinase and peroxidase activity, and synthesis of phytoalexin and other secondary metabolites) (Van Loon *et al*., 1998; Siddiqui and Mahmood, 1999; Ramamoorthy *et al*., 2001; as in Tian *et al*., 2007; Osman *et al*., 2012).

It is fortunate that some rhizobacteria may have a two-fold beneficial goal. For example, *Rhizobium etli* is one of the many soil-living bacteria able to live in conditions of nitrogen limitation due to its distinctive ability to settle on to root nodules of legumes. This bacterium, like other rhizobia, has the unique ability to form a symbiotic relationship with specific legume hosts; the host plant benefits by being provided nitrogen in the form of ammonia fixed by the bacteria, while the bacteria is provided with carbon and nutrients from the host. Moreover, *Rhizobium etli* G12 could effectively suppress the potato cyst nematode (*Globodera pallida*) and RKN (*M. incognita*) infection (Cook *et al*., 1995; Hallmann *et al*., 2001).

Organic farming practices and advanced agricultural systems usually rely on biological control as one of their components for pest control. Therefore, a great deal of research work on nematophagous bacteria is ongoing. For example, micropropagated banana plantlets are used in plantations for their genetic uniformity and pest- and disease-free condition; however, there are reports that they are very susceptible to phytonematodes. Hence, Lopes *et al*. (2011) evaluated *in vitro* colonization by ten rhizobacteria isolates of roots and rhizomes from rhizobacteria-treated 'Prata-Anã' banana (*Musa* spp.) explants, and the effects of such treatment on infection by *Meloidogyne javanica* and plantlet development. The explants were soaked in the bacterial suspension (control: saline solution) for 15 min and were transferred to medium (Phytagel). Colonization of roots and rhizome was evaluated after 30 days for the presence of a whitish halo over or around them. For the greenhouse test, the explants treated with four of the isolates were cultured on Murashige and Skoog medium. After 30 days the plantlets were transferred to tubettes and acclimated for 30 days, when they were transplanted in pots. After 24 h the soil was infested with 3000 eggs of *M. javanica* and after 60 days the plants and nematode parameters were evaluated. None of the rhizobacteria colonized roots. *Bacillus pumilus*-1, *B. pumilus*-3, *B. pumilus*-10 and *Bacillus* sp.-36 colonized the rhizomes. The other isolates caused rhizome and pseudostem necrosis and plant death. *B. pumilus*-76 increased shoot dry weight. *B. pumilus*-1, -3 and -76 reduced the nematode population in roots (Lopes *et al*., 2011). The most aggressive root parasite

affecting vines in Chile is controlled mainly chemically, and therefore rhizobacteria previously isolated from grapevine roots growing in soils with low nematode population density were evaluated to determine the effect of bacterial supernatants on nematodes collected from vineyards in *Xiphinema index*-naturally infested soils (Aballay *et al*., 2011). Rhizobacteria (38 isolates) cultivated on half-strength Tryptic Soy Broth (TSB) medium (15 g/l) with a cell suspension concentration adjusted to 10^6 CFU/ml were placed into glass Petri plates (1.5 ml of culture supernatant) along with about 50 *X. index*. Results showed that on immersion of nematodes in the culture filtrate, most of the bacterial metabolites caused nematode death. Mortality determined in the filtrates occurred with almost all bacterial isolates, as 30 and 35 isolates gave results that differed from the control (TSB) in test 1 and 2, respectively. The most nematicidal effects were produced by isolates of *Bacillus mycoides*, *Pseudomonas putida* and *Stenotrophomonas* sp. These rhizobacteria appeared to be able to suppress nematode populations and may be useful in biological control programmes in vineyards. Aballay (2012) stressed that the introduction in soil of selected microorganisms such as root rhizobacteria isolated from plants with low nematode infestation levels represents innovative alternatives to chemical nematicides. Ribeiro *et al*. (2012) evaluated the effect of bacteria isolated from a banana plantation in Brazil on mortality and motility of *Meloidogyne javanica*-J_2. Higaki and Araujo (2012) added *B. subtilis* and a chemical nematicide (abamectin) to cotton seeds to test their efficacy on plant growth in PPN-infested soils. The samples were gathered from soil under cotton cultivation infested with two PPN species *Pratylenchus brachyurus* and *R. reniformis*. The leaves of 'Deltaopal', a highly susceptible cotton genotype, could be characterized by higher proline content. Therefore, it was concluded that *B. subtilis* could work against both nematode species. Moreover, the cotton seeds treated with *B. subtilis* showed tolerance against the nematodes to the same level as abamectin. However, the cotton genotypes reacted in different way to the added chemicals and biologicals. The higher the nematode infestation in soil the more the nematode population suppression and host growth enhancement were attained. Corrêa *et al*. (2012) tested *in vitro* the effects of rhizobacteria individually and in combination on *M. incognita* (J_2) on common bean cultivars. They measured the bacterial effect on hatching and motility of the J_2 using root exudates extracted from plants whose seeds were microbiolizated. *In vitro* assays showed that combined treatment induced more favourable reduction in the tested parameters of *M. incognita*-J_2.

Moreover, the use of chemical substances produced by plant growth-promoting bacteria has proven to be a beneficial strategy to decrease various stresses that affect plant health and growth. Nascimento *et al*. (2012) reported the use of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase-producing bacterium *P. putida* UW4 as a potential biocontrol agent for pine wilt disease (PWD) induced by the pine wilt nematode (PWN) *B. xylophilus*, which is responsible for the devastation of worldwide pine forestlands; to the present time, the only means to face this serious disease lies in the eradication of *B. xylophilus*-infected trees. Such a solution is neither economic nor ecologically acceptable. A root inoculation with the bacteria *P. putida* UW4 wild-type and ACC deaminase mutant strains was performed in 3–4-month-old *Pinus pinaster* (maritime pine) seedlings followed by stem inoculation of *B. xylophilus*. The treatment alleviated *B. xylophilus*-induced symptoms and enhanced seedling growth. However, a bacterial mutant that did not produce ACC deaminase was unable to enhance the seedling development or to reduce *B. xylophilus*-induced symptoms. A strategy to induce *B. xylophilus*-resistant pine trees may be developed based on the inoculation of ACC deaminase-producing bacteria. This was the first demonstration on the utilization of such a compound as a bionematicide for tree diseases (Nascimento *et al*., 2012).

The decrease in phytonematode populations under anaerobic conditions, such as soil amendments, flooding, and tarping, may be attributed to the fermentative metabolites produced by soil bacterium. To produce these metabolites outside the soil environment, anaerobic fermentation chambers were prepared with bioglycerin from biodiesel production and urea with soil and water in 121 l barrels

(Klapp *et al*., 2012). After fermenting for 3 weeks, supernatant from these chambers was tested for herbicidal and nematicidal properties in greenhouse pots, microplots and polyethylene-covered vegetable beds. Soils drenched with supernatant from anaerobic chambers had decreased *M. incognita* and *R. reniformis* populations, and increased beneficial nematode and fungi populations. Positive growth response and increased yield occurred in 'Marketmore 76' cucumber (*Cucumis sativus*), 'Tiny Tim' tomato (*Solanum lycopersicum*) and 'Conqueror III' squash (*Cucumis pepo*). Kavitha *et al*. (2012) found that the endospore-forming rhizobacterium *B. subtilis* used as a model system for gram-positive organisms produced more than 24 lipopeptide antibiotics, hydrolytic enzymes and other secondary metabolites. Three families of *Bacillus* lipopeptides – surfactins, iturins and fengycins – are being extensively tested as good candidates against numerous taxa of phytopathogens such as bacteria, fungi and nematodes. Since *M. incognita* is among the most damaging pathogens attacking a wide range of crops, its destructive impact on noni (*Morinda citrifolia* L.), a plant considered as a panacea to cure many diseases, is not unusual. This nematode parasite causes severe root infections in noni that reduce plant growth and ultimately lead to death of the plant. Kavitha *et al*. (2012) isolated six antagonistic endophytic strains of *B. subtilis* (Bs N 1, Bs N 3, Bs N 4, Bs N 7, Bs 5 and Bs N 11) from the noni fruits and plant tissues. An existing strain Bbv 57 isolated from banana proven for its nematicidal action was included in the study. *B. subtilis* strain Bs 5 with high surfactin and iturin activity has proven its ability to suppress *M. incognita in vitro*. The study revealed that the crude antibiotic exerted maximum lethal effect on RKN by inhibiting egg hatching and causing juvenile mortality. Juveniles (J_1) inside the eggs were paralyzed and killed due to the antagonistic effect of the antibiotic and thereby proving its larvicidal action, which is irreversible. SEM analysis of RKN eggs after 48 h of treatment with antibiotic revealed the deposition of antibiotic over the nematode eggs, which resulted in the death of the eggs due to its ovicidal action. Translocation and colonization potential of these effective endophytic strains of noni were studied by radio labelling method using the isotope 32P and trace analysed by autoradiography. The success of *B. subtilis* is due to the genes encoding surfactin and iturin synthesis and hence it can be a valuable agent for managing RKN in noni. Abdelmoneim and Massoud (2012) found many bacterial species in Egyptian soil which thrive in the plants' rhizosphere or around plant tissues and could stimulate plant growth and decrease PPN population as antagonistics. Such bacteria are conjointly called PGPR. They tested the effect of six bacterial species from Egyptian soil, i.e. *P. putida*, *P. florescens*, *P. marcescens*, *Bacillus amyloliquefaciens*, *B. subtilis* and *B. cereus*, related to PGPR, on tomato plant growth and RKN multiplication 45 days after nematode infection. The highest shoot dry weight (43.00 g) was detected in the plants treated with *S. marcescens*, followed by *P. putida* (34.33 g), *B. amyloliquefaciens* (31.66 g), *B. fluorescens* (30.0 g), *B. subtilis* (29.0 g), *Bacillus cereus* (27.0 g) and nematode alone (untreated) 20 g/plant. The highest plant height was obtained from treating with *S. marcescens*, *P. fluorescens*, *P. putida*, *B. amyloliqufaciens* and *P. putida*, but no significant differences occurred between treatments. The highest number of fruits per plant was observed in plants treated with *S. marcescens* (10.66), followed by *B. amyloliqufaciens* (8.66), *P. putida* (8), *P. fluorescens* (8) and *B. cereus* (7.66). No significant differences were found between the last four treatments. Plant yield (g) was the highest with *S. marcescens* (319.6 g/plant) and the lowest in plants with nematode alone (untreated). The lowest number of J_2/10 g of soil (78), galls/root (24.33) and egg masses/root (12.66) were associated with *S. marcescens* treatment. Ali (2012) isolated several bacteria and actinomycetes from *Meloidogyne* spp. egg masses and *Heterodera zeae* cysts in Egypt. Screening the isolates for nematoxic effects resulted in four species with antagonism to PPN. The isolates were *Clostridium butyricum*, *Desulfovibrio desulfuricans*, *P. fluorescens* and *Streptomyces anulatus*. They were frequently associated with both egg masses and cysts. Liquid cultures (LCs) of the four isolates at concentrations as low as 0.2% inhibited hatching of *M. javanica* eggs. The culture at a concentration of 0.6% was highly toxic to juveniles of *M. javanica*

and *R. reniformis*. All the tested microorganisms were responsible for considerable reduction in root and soil populations, maturation and reproduction of both nematodes.

9.2.4 Cry protein-forming bacteria

Bacillus thuringiensis (Bt) is a bacterium that forms parasporal crystals during the stationary phase (Schnepf *et al*., 1998). Bt produces one or more parasporal crystal inclusions (Cry protein or δ-endotoxins), which are well known as toxins to many pest groups (Feitelson *et al*., 1992; Bernhard *et al*., 1997; Schnepf *et al*., 1998; Maagd *et al*., 2001; Tian *et al*., 2007). Some Cry proteins are also toxic to phytonematodes (Prasad *et al*., 1972; Wei *et al*., 2003; Mohammed *et al*., 2008; Abdelmoneim and Massoud, 2009; Khan *et al*., 2010). Abdelmoneim and Massoud (2009) found that the spherical crystal toxin gave the highest reduction in nematode population because they can easily pass through the nematode mouth part. These proteins are pore-forming toxins that are lethal against insects and some phytonematodes (Abd-Elgawad, 1995; Maagd *et al*., 2001). Nematicidal activity has been found in several families of *B. thuringiensis* proteins, such as Cry5, Cry6, Cry12, Cry13, Cry14, Cry21 and Cry55 (Guo *et al*., 2008; Zhang *et al*., 2012). For example, such proteins expressed in transgenic roots significantly suppressed *M. incognita* reproduction by Cry6A whereas Cry5B decreased the *M. incognita* gall number and reduced progeny levels by nearly three-fold (Li *et al*., 2007, 2008). Guo *et al*. (2008) and Yu *et al*. (2008) isolated several specific *B. thuringiensis* strains that showed high nematicidal activity against PPN. Subsequently, such nematicidal proteins encoding genes *cry6Aa2*, *cry5Ba2* and *cry55Aa1*, were isolated from the highly nematicidal *B. thuringiensis* strain YBT-1518. A synergism between Cry6Aa and Cry55Aa was proved by Peng *et al*. (2011) against *M. incognita*. Also, using UV irradiation, three Bt mutants could generally result in better control of *M. incognita* than the wild type, in terms of numbers of nematode juveniles in soil, galls and egg masses (Eissa *et al*., 2010). Moreover, the three mutants improved growth of sunflower plants compared to the wild type. Because investigating whether or not specific crystal proteins can enter RKNs would assist to define the molecular exclusion limit and would expedite the design of a transgenic resistance method to control RKNs (Urwin *et al*., 1997), the pathway of *B. thuringiensis* crystal proteins entering *Meloidogyne hapla* (J_2) by confocal laser scanning microscopy (CLSM) was monitored and assimilation efficiency of the crystal proteins by the *M. hapla* (J_2) was tested by enzyme-linked immunosorbent assay analysis (ELISA) (Zhang *et al*., 2012). The uptake capacity of the crystals by the RKN, i.e. *M. hapla* (J_2), was reduced with mounting of the crystals' molecular mass. Thus, Zhang *et al*. (2012) concluded that molecular mass of such crystals may be exploited in developing a basis for a transgenic resistance approach to manage *Meloidogyne* spp. Such nematicidal and insecticidal crystals of Bt were recorded to demonstrate similar modes of action (Abd-Elgawad, 1995). The crystals usually induce degradation and gaps in the cell membrane of gut epithelial cells (Crickmore, 2005). The target PPN larvae swallow them, and thereafter the crystals dissolve within the PPN gut, and this is pursued by proteolytic activation. Their toxicity is most effective on the cells of the mid-gut (Marroquin *et al*., 2000; Tian *et al*., 2007). This has led to their use as insecticides, and more recently to genetically modified crops using Bt genes. However, many crystal-producing Bt strains do not have insecticidal properties (Regnault-Roger, 2012).

9.2.5 Endophytic bacteria

Endophytic bacteria are always found internally in root, and to a less extent in stem tissue, where such organisms can persist in most plant species. This group may be encountered in fruits and vegetables, and are present in both stems and roots, but do no damage to the inhabited host. They may be regarded as environmentally friendly organisms since they usually promote plant growth and suppress disease development and PPN (Tian *et al*., 2007). Being capable of occupying internal host tissues, endophytic bacteria are valuable tools to improve crop yield and antagonize PPN.

By systemically occupying roots, they have potential to work against PPN. This is because they: (i) are easily cultured *in vitro*; (ii) have the privilege of application as seed treatment; (iii) can reduce initial damage to plant roots; (iv) can get rid of competition with other microbes and can also modify host's response to PPN attack; (v) can enhance growth of the colonized plant but do not cause phytotoxic symptoms; and (vi) can make use of root exudates for multiplication (Siddiqui and Shaukat, 2003). Munif *et al*. (2000) isolated 181 endophytic bacteria and tested them against PPN. They found antagonistic properties towards *M. incognita* in 21 of the bacteria. Some species of these bacteria were also documented to have activity against *P. penetrans*. Among them, *Microbacterium esteraomaticum* and *Kocuria varians* have been shown to play a role in root-lesion nematode control through the weakening of host proliferation, but without inducing any yield reduction (Sturz and Kimpinski, 2004). Although rhizobacteria and endophytic bacteria have different microhabitats, both groups display some common mechanisms for enhancing plant growth and managing phytopathogens, e.g. niche competition with the harmful pathogen/parasite, manufacture of chemicals that suppress pathogen/parasite activity and induction of systemic resistance (ISR) in their hosts (Hallmann, 2001). However, there are still some cases in which endophyte status does not affect phytonematodes. For example, Nyczepir (2011) evaluated tall fescue grass (*Schedonorus arundinaceus* (Schreb.) Dumont) cultivars with or without endophytes for their host suitability designation to *Mesocriconema xenoplax* and *Pratylenchus vulnus* in the greenhouse. They found that peach (*Prunus persica*) supported greater ($P \leq 0.05$) reproduction of *P. vulnus* and *M. xenoplax* than all tall fescue cultivars. Furthermore, all tall fescue cultivars were rated as poor hosts for *P. vulnus* and good hosts for *M. xenoplax* based on nematode reproduction factor. Root endophyte status was independent from nematode reproduction and host susceptibility.

On the contrary, recent work published on bacteria associated to *B. xylophilus* showed that in areas recently invaded by this nematode, healthy pine trees of the species *P. pinaster* have a diverse endophytic microbial community (Morais *et al*., 2011). From these areas, nematodes isolated from infected trees carried associated bacteria that belonged to different species according to the area where the nematodes were isolated. Consequently, the possibility of a protective role to the tree, by the endophytic microbial community, against the nematode has become a hypothesis. In order to test it, isolated bacteria associated to the nematodes infecting *P. pinaster* were tested for their ability to kill *B. xylophilus*, *in vitro*. Tested strains more active in killing nematodes were selected and the bacterial products, produced during development, investigated with an end in view to determine their nematicidal activity. This nematicidal activity was analysed for 46 strains. Only seven bacterial strains did not show toxicity against *B. xylophilus* and the only genus with all the strains being non-toxic was *Burkholderia*. All strains of the genus *Pseudomonas*, except one strain of *P. putida*, showed toxicity against the nematodes. The genus *Serratia* included the more toxic strains to the nematodes: all except one showed the highest toxicity level. *Serratia* strains were screened for the different products that could interact with the nematode. Major products identified with potential biotechnological use were serrawettin and proteases (Morais *et al*., 2011). Following the same line of thinking, Paiva *et al*. (2011a) noticed that healthy pine trees *P. pinaster* contain a diverse endophytic microbial community in Portugal, and so the nematicidal ability was analysed for strains associated to nematodes from trees with PWD. Screening of these isolates revealed the presence of bacterial strains producing different extracellular products. Strains more toxic to the nematodes belonged also to the genus *Serratia*. Strains from *S. marcescens* have already been isolated in association with *B. xylophilus* (J_2) from *P. densiflora* collected in Korea. Paiva *et al*. (2011b) could identify and characterize the hydrolytic enzymes produced by the *Serratia* isolates and assess their nematicidal activity. The toxicity assays revealed that only two fractions, separated by FPLC, showed nematicidal activity compared to the whole extract. These fractions showed higher toxicity rates and included different concentrations of two different proteases.

The use of inhibitors selective to serine proteases or to metalloproteases demonstrated that serine protease was mostly in charge of the supernatant toxicity. They concluded that understanding the infection process and relevant factors should be attained to effectively control *B. xylophilus* dispersion. Proença *et al*. (2011) also assessed the microbial community associated with the PWN and with other nematodes isolated from *P. pinaster* with PWD from three different affected forest areas in Portugal. Bacterial strains (143) were isolated from nematodes collected from 14 *P. pinaster* trees. The bacterial strains were identified by 16S rRNA gene partial sequence. Most predominant genera were *Pseudomonas* and *Burkholderia* belonging to the family Enterobacteriaceae. Highly encountered species included *Pseudomonas lutea*, *Yersinia intermedia* and *Burkholderia tuberum*. Most populations of the bacteria associated with the nematodes differed based on the forest area and none of the isolated bacterial species was detected from all forest areas. The isolates produced 60–100% of siderophores and at least 40% produced lipases that enable these bacteria to play a role in plant physiological response. They documented the diversity of the microbial community in association with *B. xylophilus* and other nematodes isolated from *P. pinaster* with PWD (Proença *et al*., 2011). Much more investigation on the bacteria associated with *B. xylophilus* is needed to exploit them in a possible tactful management method of PWD.

9.2.6 Symbiotic bacteria

There are two major groups of bacteria/entomopathogenic nematodes (EPN) symbiotic relationship. These are *Xenorhabdus* spp./*Steinernema* spp. and *Photorhabdus* spp./*Heterorhabdus* spp. (Paul *et al*., 1981; Askary, 2010). Once within the insect host, these bacteria are released by EPN to kill the insect and provide a suitable nutrient environment for nematode reproduction (Boemare *et al*., 1997; Askary, 2010). The interaction between the symbiotic complex and PPN has been receiving a great deal of research in field, greenhouse and laboratory studies (e.g. Bird and Bird, 1986; Grewal *et al*., 1997, 1999; Perry *et al*., 1998; Abd-Elgawad and Aboul-Eid, 2001a,b; Lewis *et al*., 2001; Lewis and Grewal, 2005; Abd-Elgawad *et al*., 2008, 2013a,b). The topic of whether or not EPN represent an alternative to currently used PPN management methods remains controversial (Lewis and Grewal, 2005). Of the six species of EPN tested against various species of PPN, all have caused some reduction in PPN numbers or reproduction in at least one trial. Members of both genera of EPN have yielded positive and negative results. Only *S. feltiae* has caused some suppression in every case where it was tested against PPN (Lewis and Grewal, 2005). The latter authors speculated that the foraging strategy of *Steinernema carpocapsae* has resulted in relative poor performance in these assays due to the fact that they are usually located in the upper 2 cm of soil. Moreover, of the seven papers, checked by Lewis and Grewal (2005), that have a RKN as one of the targets by EPN, suppression was recorded in all but a single case. These studies varied significantly in the application methods used, and the results from studies that have extremely high doses at multiple times may be of limited value due to high dose costs. Also, in two of the three studies, examined by Lewis and Grewal (2005), *Pratylenchus penetrans* population was not reduced. This demonstrates a desperate need to know more about how EPN applications impact soil ecology.

On the other hand, the bacterium-free filtrates of different Egyptian *Photorhabdus* isolates were evaluated by mycelial plug on NBTA plates and paper disc diffusion assays for their antifungal and antibacterial activity, respectively, against 15 soil microorganisms (Abd El-Zaher and Abd-Elgawad, 2012). The phase I variant of *Photorhabdus* could inhibit growth of all tested fungal species to an extent that varied considerably with both the fungal species/strain and the isolate of the *Photorhabdus* bacterium, 9 days after incubation. In addition, six out of the ten symbiotic bacterial isolates/strains demonstrated antibacterial activities; each one of the six *Photorhabdus* isolates/strains could inhibit growth of one, two, or three soil bacterial species tested. Such effects of these symbionts on fungi and bacteria might imply parallel PPN inhibition.

Also, the effect of bacterium-metabolite concentration for each of these isolates under some physical parameters on the metabolite-induced mortality for the greater wax moth, *Galleria mellonella*, larvae and the magnitude of the bacterial growth was investigated (Abd El-Zaher *et al*., 2012). The knowledge gained from the impact and activity of these symbionts under such naturalistic parameters tested may be exploited to develop and optimize the utilization of such bacteria and their products as biocontrol agents against pests including phytonematodes. This is especially important since a potentially antagonistic effect of the symbiotic complex on PPN has been reported (e.g. Bird and Bird, 1986; Grewal *et al*., 1997, 1999). Further studies indicated that these bacteria are in charge of PPN suppression (Samaliev *et al*., 2000). Several types of their secondary metabolites work as the nematicidal agents: ammonia, indole and stilbene derivative (Hu *et al*., 1997, 1999). They were poisonous to eggs and J_2 of *M. incognita* and to J_4 and adults of PWN (Hu *et al*., 1999; as in Tian *et al*., 2007). Recent studies with isolates of EPN (families Steinernematidae and Heterorhabditidae) showed a clear antagonistic activity on the false root-knot nematode, *Nacobbus aberrans*, which would be caused by the EPN-symbiotic bacteria (SB) complex (Lax *et al*., 2012). The effect of SB extracted from EPN on the PPN was evaluated *in vitro* and on tomato plants. A nematicidal effect (under *in vitro* conditions) on J_2 was observed, producing 100% mortality in some cases. The application of SB and their metabolites to the soil significantly reduced the number of galls (33% to 67%) and nematode reproduction (55% to 80%) on the host. These results show that SB and their metabolites might be used as potential biological control agents of local *N. aberrans* populations. Also, Nethi *et al*. (2012) found that inundative field applications of EPN have no significant long-term adverse impact on non-target arthropods, but improvements in plant growth were observed in fields treated with EPN in some areas. This plant growth improvement was attributed to the decrease in abundance of PPN following application of EPN.

Currently, the interaction between EPN and PPN has become a subject of intense study from both the ecological and commercial perspective. While suppression of PPN, though a non-target effect, is considered beneficial from the pest management perspective, concerns were raised about its mechanisms and the possible adverse impact of EPN on other nematode trophic groups in soil and ecosystem services they provide (Tian *et al*., 2007). Nethi *et al*. (2012) unravelled a unique phenomenon of selective suppression of PPN by EPN without any adverse impact on beneficial free-living trophic groups of nematodes in soil food webs. This effect was referred as a beneficial non-target effect of EPN. Abd-Elgawad *et al*. (2008, 2013a) summarized possible mechanisms of PPN suppression caused by this symbiotic complex. Recent studies have also demonstrated that EPN and their symbiotic bacteria can induce systemic resistance in plants, which may act against PPN (Nethi *et al*., 2012). Activities of some antioxidant enzymes in roots of plants hosting the bacteria and/or PPN were investigated (e.g. Molinari and Abd-Elgawad, 2007; Abd-Elgawad and Kabeil, 2010, 2012) and might further be a good tool to give an insight into how EPN could, possibly selectively, suppress PPN in the soil ecosystem. Abd-Elgawad *et al*. (2008) reported that EPN may be used for a two-fold goal, i.e. controlling PPN and insect pests simultaneously. Yet, to achieve the first goal necessitates optimal application tactics of EPN such as the delivery of the EPN-dauer stage juveniles near the plant roots for effective PPN control (Abd-Elgawad *et al*., 2013a).

9.3 Nematophagous Bacteria Interaction with Biotic and Abiotic Factors

Nematophagous bacterium density and species within a given environment is not constant and changes over time due to their interaction with their surrounding biotic and abiotic factors. The understanding and manipulation of factors affecting these bacteria, their nematode hosts and accompanied plants is imperative for tactful use of these bacteria (e.g. Chen and Dickson, 1998; Siddiqui and

Mahmood, 1999; Dong and Zhang, 2006; Stirling, 2014). Major factors influencing their total population densities include their characteristics and microenvironment's endemic biomass, plant age and type, climate, soil properties as well as various other biotic and abiotic factors. The effects of some of these factors have been reported (e.g. Siddiqui and Mahmood, 1999; Siddiqui and Shaukat, 2003; Abd El-Zaher *et al*., 2008, 2012; Gowen *et al*., 2008; Luc, 2009; Crow *et al*., 2011; Luc *et al*., 2011a,b). Admittedly, the use of this bacterial group as a bionematicide against PPN depends not only on their antagonistic effect but also on such biotic and abiotic factors. For example, the bacterium *P. penetrans* is cosmopolitan, frequently found in different climates and environmental conditions, which suggests utilization as a promising candidate for the biocontrol of RKNs (Gowen *et al*., 2008), but host specificity (Kumari and Sivakumar, 2006; Davies *et al*., 2008) and infection only on nematode juveniles are some of the limiting factors for this bacterium to be used as a biocontrol agent (Chaudhary and Kaul, 2011). However, its combination with other microbial agents, organic amendments or chemical nematicides has shown a cumulative effect on its performance (Curto, 2006; Kumari and Sivakumar, 2006; Javed *et al*., 2008; Kumar, 2008). Studies conducted by Chaudhary and Kaul (2011) on *M. incognita*-infected chilli (*Capsicum annuum* L.) revealed that *P. penetrans* + *Paecilomyces lilacinus* are more favoured for both nematode control and crop yield, which corroborated other findings of Sosamma and Koshy (1997). Also, the invasion and infectivity of *P. javanica* (J_2) encumbered with spores of *P. penetrans* are influenced by the temperature and the time J_2 are in the soil before exposure to roots (Giannakou and Gowen, 2004). At 30°C the suppression in infection by spore encumbered J_2 was greater than at 18°C. The bacterium could multiply exponentially between 21 and 36°C and the base for bacterial development was estimated by extrapolation to be 18.5°C. In addition, several soil variables such as soil texture, moisture and temperature, as well as *P. penetrans* spore densities and nematode age were tested in the laboratory for their effects on spore attachment to *M. incognita* juveniles (Talavera and Mizukubo, 2003). In a clay-loam soil, 100% attachments could be attained at densities of 5×10^5 spores/g soil and above. Attachment was higher in loamy-sand than in sandy-loam and clay-loam soils, but lower when soil moisture was under 10% than when it was over 25%. Numbers of juveniles with spores attached were greater when soil temperatures were 25°C and 35°C than at 15°C and lower in 7–30-days-old juveniles than in 0–6-days-old juveniles. All factors that favoured nematode mobility in soil increased *Pasteuria* spore attachment to *M. incognita* juveniles (Talavera and Mizukubo, 2003). As for opportunistic parasitic bacteria, *B. laterosporus* is a ubiquitous species that has been isolated from a wide range of materials including soil, gemstones, lahar, fresh water, sea water, insect bodies, leaf surfaces, locust beans, compost, milk, cheese, honey, starchy foods, gelatin-factory effluents, animal hide and wool, and quails (Ruiu, 2013). *B. laterosporus* bioactivity can adversely affect other organisms in its microhabitat such as insects, nematodes, algae, bacteria and fungi due to the production of various metabolites. As for endophytic bacteria, the internal plant tissues may work as a protective environment. Nonparasite rhizobacteria usually reduce PPN populations by occupying the rhizosphere of the host plant. Breen (1994) idealized a model with several soil physical and chemical factors affecting the changes in plant resistance to insect pests in the presence of endophytes. Numerous factors that affect the endophyte's host will also impact these bacteria (Hallmann *et al*., 1997). Quantification of *Acetobacter diazotrophicus* has demonstrated that addition of nitrogen fertilizer with high levels of nitrogen resulted in a severe decrease in the bacterial numbers. Therefore, fertilization with high nitrogen levels may also work as a double-edged sword in such a case (Siddiqui and Shaukat, 2003). Mohammed *et al*. (2008) reported that applications of formulated Bt are not toxic to most beneficial or predator insects as well as birds and fish. No evidence exists that Bt causes teratogenic effects in mammals. Genetically engineered Bt plants should consider high selectivity and a linear dose-response in the introduced toxicity. These two traits, selectivity and efficacy, can be agitated by synergism. This latter can provoke unforeseen

and harmful effects on non-target organisms. Therefore, some researchers suggested that systematic research should be done on synergism between Bt toxins and potential extrinsic operators. This type of research should become a precondition for evaluating Bt plants including their effects on both beneficial and harmful nematodes. Some biotic and abiotic factors affecting the above-mentioned *Xenorhabdus* spp. and *Photorhabdus* spp., symbionts of the EPNs were studied (Abd El-Zaher *et al*., 2012) since these bacteria have been found to be toxic to several PPN (e.g. Grewal *et al*., 1997, 1999; Hu *et al*., 1999; Samaliev *et al*., 2000; Lax *et al*., 2012). Yet, more investigations were done on the effects of their toxins and filtrates on insect pests, which may imply parallel influence on PPN. For example, when different *Photorhabdus* spp. were grown in yeast salt broth in the absence of EPNs, the bacteria produced a toxin protein that is lethal when fed to *G. mellonella* larvae. Broth cultures of five isolates of *Photorhabdus* (A, B, C, D, E) showed various mortality rates to the insect larvae when different concentrations (5, 10 and 20 ml) from bacterial cell suspension were mixed with two kinds of media (wheat bran and fine sand) as compared to broth alone (control); after 1 day the larval mortality reached 100% on sand inoculated with 20 ml cells suspension of the isolates A, C and D (Abd El-Zaher *et al*., 2008). These bacterial cells can enter in the haemocoel in the absence of EPN vector. Hence, the authors suggested including such bacteria in the IPM programmes. The effect of metabolite concentration for each of these bacterial isolates and some physical parameters (i.e. temperature, pH and sodium chloride) on the metabolite-induced mortality for the insect larvae and the magnitude of the bacterial growth under different concentrations of NaCl and pH values were also studied. This mortality varied ($P \leq 0.05$) among the tested Egyptian isolates at the ranges assigned for these physical parameters. The cell-free filtrates of 4×10^7 cells/ml broth of the bacterium *Photorhabdus luminescens akhurstii* isolate B were able to attain full insect mortality after 1 day of exposure, demonstrating the highest virulence against *G. mellonella* larvae. Moreover, growth of this bacterium was superior to that of all others in broth with 0.3% NaCl. The higher the metabolite dose the more was the percentage mortality of *G. mellonella* recorded up to 10 days of exposure (Abd El-Zaher *et al*., 2012).

9.4 Conclusions and Future Prospects

Accelerating public concern about overuse of chemical nematicides has sparked wide concern to develop environmentally friendly biological alternatives. Over the past three decades, a large number of studies have been conducted to investigate the benefits of microorganisms as biocontrol agents against PPN. Researchers have been studying more and more bacteria as pathogens of PPN and have shown different mechanisms leading to suppression effects on nematode pest populations. The nematophagous bacteria include the categories listed in Table 9.1. Not surprisingly, as with other biocontrol agents, nematophagous bacteria have their merits and demerits for management of phytonematodes. Usually, they are safe and their harmful residues are not detected or may be biodegradable, they can be cheaper than chemical nematicides when locally produced, and they may be used against PPN with a clear aim at the avoidance of environmental pollution and health hazards of chemical nematicides. On the other hand, some bacterial species/strains may have high specificity, as in *Pasteuria*, which may require an exact identification of the nematode pest and the use of multiple products to be used; although this can also be an advantage in that the biopesticide is less likely to harm species other than the target. The bacteria often have slow speed of action (thus making them unsuitable if a pest outbreak is an immediate threat to a crop), and they often show variable efficacy due to the influences of various biotic and abiotic factors on their efficacy and reproduction. Yet, living organisms, like bacteria, usually seek adaptation to their surrounding niche in terms of biological, chemical, physical or any other form of control. If the target population is not exterminated or rendered incapable of reproduction, the surviving population can acquire a tolerance of whatever pressures are brought

Table 9.1. Reported bacterial groups with pathogenic activity against plant-parasitic nematodes (adapted from Tian *et al*., 2007).

Nematophagous bacterial group	Genus and species	Target nematodes	Pathogenic effects on nematodes	Action mode	References
Parasitic bacteria	Four species: *Pasteuria penetrans*; *P. thornei*; *P. nishizawae*; *Candidatus* Pasteuria usgae	323 nematode species of 116 genera	Major economic important plant-parasitic nematodes have been observed to be parasitized by *Pasteuria*	Parasitism	Chen and Dickson (1998); Siddiqui and Mahmood (1999); Bekal *et al*. (2001); Giblin-Davis *et al*. (2001, 2003); Bird *et al*. (2003); Crow *et al*. (2011)
Opportunistic parasitic bacteria	*Bacillus nematocida* (*Bacillus* sp. B16); *Brevibacillus laterosporus*; *Bacillus* sp. RH219 etc.	*Panagrellus redivivus* and *Bursaphelenchus xylophilus*	*B. laterosporus* strain G4 could penetrate the nematode (*P. redivivus* and *B. xylophilus*) cuticles and eventually digest the target organism in the laboratory	Parasitism, production of enzymes and toxin	Huang *et al*. (2005); Niu *et al*. (2005, 2006); Tian *et al*. (2006, 2007); Ruiu (2013)
Rhizobacteria	Distribution in more than 29 genera. *Bacillus* (more than 15 species) and *Pseudomonas* (more than 11 species) are two of the most dominant populations	Reduce nematode populations in soil	Different rhizobacteria showed different degrees of suppression on nematodes in various conditions. Three commercial bionematicides from bacteria all belong to this group	Interfering with recognition, production of toxin, nutrient competition, plant-growth promotion; induction of systemic resistance	Cronin *et al*. (1997); Siddiqui and Mahmood (1999); Jonathan *et al*. (2000); Mena and Pimentel (2002); Meyer (2003); Abally *et al*. (2011); Corrêa *et al*. (2012); Ribeiro *et al*. (2012)
Parasporal crystal-forming bacteria	*Bacillus thuringiensis* (Cry5, Cry6, Cry12, Cry13, Cry14, Cry21)	Root-knot nematode, *Trichostrongylus*; *Colubriformis*, *Caenorhabditis elegans* and *Nippostrongylus brasiliensis*	These Cry proteins showed toxicity to larval stages of free-living and parasitic nematodes	Cry proteins caused damage to the intestines of nematodes	Abd-Elgawad (1995); Marroquin *et al*. (2000); Wei *et al*. (2003); Eissa *et al*. (2010)
Endophytic bacteria	The majority of rhizobacteria can also be identified as endophytic bacteria	Root-knot nematode and root-lesion nematode etc.	Suppress root-knot nematodes and root-lesion nematode etc.	Rhizobacteria and endophytic bacteria use some of the same mechanisms	Munif *et al*. (2000); Hallmann (2001); Siddiqui and Shaukat, (2003); Morais *et al*. (2011)
Symbiotic bacteria of entomopathogenic nematodes	Two genera: *Xenorhabdus* and *Photorhabdus*	Root-knot nematode, pine wilt nematode and root-lesion nematode etc.	Toxic to nematodes and inhibit egg hatch	Toxin production (ammonia, indole and stilbene derivative)	Bird and Bird (1986); Grewal *et al*. (1997, 1999); Hu *et al*. (1997, 1999); Perry *et al*. (1998); Samaliev *et al*. (2000); Lewis *et al*. (2001); Abd-Elgawad *et al*. (2008)

to bear, resulting in an evolutionary arms race. The growth of such beneficial bacteria is often unpredictable and too variable for large-scale implementation (Meyer, 2003; Crow *et al*., 2011; Luc *et al*., 2011b).

Therefore, further fundamental and applied studies to explore the interactions among the bacterial strains, nematode target, soil microbial community, physical and chemical factors of soil, plant and environment will probably formulate a clearer picture for tactful and successful exploitation of these bacteria and bolster their role in the management of PPN in various agriculture systems. Generally, an efficacy gap is found between the outcomes of biocontrol agents under laboratory and field conditions. Therefore, to strengthen their field efficacy, sustainable working methodologies have been proposed, including IPM (Sikora *et al*., 2005; Stirling, 2014). An increased knowledge of the molecular basis and techniques for exploring the pathogenic mechanisms of these bacteria on nematodes not only will lead to a sophisticated PPN control, but also may create novel biocontrol strategies for PPN. For example, chemotactic factors released by the hosts or pathogens are known to be in charge of the contact between bacteria and their hosts. Therefore, knowledge of these mechanisms is needed to control PPN populations by skilful manipulation of these nematophagous bacteria and their products (Tian *et al*., 2007). Eventually, further discovery, characterization and commercial development/application of the nematophagous bacteria to control PPN are expected to significantly contribute in expanding their role in addressing the serious damage and losses of economically important crops caused by the phytonematodes.

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10 Nematophagous Bacteria: Virulence Mechanisms

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10.1 Introduction

Bacteria may affect nematode populations by a series of direct mechanisms, including parasitism and antibiosis and indirectly by interfering with the recognition of host plants, inducing systemic resistance and improving plant health (Tian *et al*., 2007). Bacteria may be classified as antagonists, parasites and symbionts, according to their ecological association with nematodes. Antagonistic bacteria are saprophytes that may use nematodes as a source of nutrients under certain conditions, but are not dependent on them for survival. These bacteria kill nematodes through the production of toxins, enzymes, volatile compounds and antibiotics. On the other hand, obligate parasitic bacteria and symbionts depend on the nematode host for survival and have evolved a biotrophic lifestyle with little or no production of enzymes and toxic compounds.

Independent of their lifestyle, bacteria that affect nematodes directly through parasitism and antibiosis need to produce pathogenicity factors that make them able to kill nematodes. In an analogy with relationships between plants and pathogens, we consider bacterial virulence factors as: (i) compounds produced by bacteria that increase their ability to kill nematodes; and (ii) compounds that are able to manipulate the defences of nematode hosts and allow bacterial growth. In most studies on the relationships between bacteria and nematodes, the concepts of pathogenicity and virulence factors are not always clearly delimited and are often used interchangeably.

In this chapter we have tried to focus primarily on bacteria pathogenic to phytonematodes. The methods required to study the pathogenicity and virulence in bacteria–nematode relationships are highlighted. Model free-living nematodes such as *Caenorhabiditis elegans* and *Panagrellus redivivus* have also been discussed.

10.2 Antagonistic Bacteria

Antagonistic bacteria, including soil, root and endophytic bacteria are commonly found in association with nematodes. Most antagonistic bacteria are opportunistic pathogens that may produce enzymes, toxins, antibiotics and volatile compounds as pathogenicity and virulence factors to kill and use nematodes as a food source (Table 10.1). Some of these bacteria, despite affecting nematode populations, do not

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Table 10.1. Pathogenicity and virulence factors produced by antagonistic bacteria against nematodes.

Bacteria	Pathogenicity/virulence factor	Effects	Target nematode	References
Bacillus nematocida B16	Alkaline serine protease	Cuticle degradation, 95% nematode death after 48 h	*Panagrellus redivivus*	Niu *et al*., 2006a,b
	Neutral protease Bae16	Purified protease could degrade the nematode cuticle within 24 h	*P. redivivus* and *Bursaphelenchus xylophilus*	Niu *et al*., 2007; Niu *et al*., 2010
	Extracellular serine protease Bace16	Degradation of nematode cuticle and 100% of mortality after 60 h	*P. redivivus*	
	VOCs that attract nematodes	Damage intestine when bacteria are ingested, 95–100% of nematode death	*Caenorhabiditis elegans*	
Bacillus sp. D2	Chitinase	Eggshell degradation	*Globodera rostochiensis*	Margino *et al*., 2012
Bacillus megaterium YFM3.25	2-nonanone; 2-undecanone decanal; dimethyl disulfide; benzene acetaldehyde	80 to 100% nematicidal activity in Petri dish assays	*M. incognita* (juvenile and egg)	Huang *et al*., 2010
Bacillus simplex, *B. subtilis*, *B. weihenstephanensis*, *Serratia marcences*, *Stenotrophomonas maltophilia*	Benzaldehyde; 2-octanol; phenol; terpineol; benzeneacetaldehyde; decanal, 2-nonanone; phenyl ethanone; 2-undecanone; cyclohexene; dimetyl disulfide; benzeneethanol; phenyl ethanone	84 to 100% nematotoxic activity in *in vitro* bioassays	*B. xylophilus* and *P. redivivus*	Gu *et al*., 2007
Bacillus thuringiensis	Cry5B; Cry5Ba2; Cry6A; Cry6Aa2; Cry55Aa1; metalloproteinase Bmp1	4-fold reduction in *M. incognita* reproduction on transgenic Cry6A-expressing on tomato plants; Bmp1 and Cry proteins caused damage to intestinal cells	*M. incognita*, *M. hapla*, *C. elegans*	Li *et al*., 2007, 2008; Guo *et al*., 2008; Luo *et al*., 2013

Continued

Table 10.1. Continued.

Bacteria	Pathogenicity/virulence factor	Effects	Target nematode	References
Brevibacillus laterosporus G4	Extracellular alkaline protease BLG4	Degradation of the cuticle and the epidermis; endospores attach to nematode cuticles	*P. redivivus* and *B. xylophilus*	Huang *et al*., 2005; Tian *et al*., 2006, 2009
	Neutral protease NPE-4	Little nematicidal activity was shown by purified NPE-4 and it was unable to degrade the intact cuticle of nematode, but addition of NPE-4 improved the pathogenicity of crude enzyme extract from wild-type *B. laterosporus*, suggesting that NPE-4 could degrade proteins of inner layer of nematode	*P. redivivus*	Tian *et al*., 2007
Lysinibacillus mangiferahumi M-GX18^T	2-octanol; cyclohexene; 3-chloro-4-fluorobenzaldehyde; dibutyl phthalate; 2-nitro-2-chloropropane; dimethachlore; weedar; dimethyl disulfide	100% nematicidal activity in bioassays under *in vitro* condition	*M. incognita*	Yang *et al*., 2012
Stenotrophomonas maltophilia G2	Serine protease	Cuticle degradation	*B. xylophilus* and *P. redivivus*	Huang *et al*., 2009
S. maltophilia MP1; *Achromobacterium* sp. UP1	Chitinase	Egg hatching inhibited	*G. rostochiensis*	Cronin *et al*., 1999
Pseudomonas fluorescens CHA0	Protease aprA; 2, 4-diacetylphloroglucinol; hydrogen cyanide	Egg hatching reduced to 45%; reduced juvenile mobility and increased juvenile mortality	*M. incognita* *M. javanica*	Siddiqui *et al*., 2005 Siddiqui *et al*., 2006
Pseudomonas fluorescens F113	2,4-diacetylphloroglucinol	Increased egg hatching by 2-fold; decreased juvenile mobility by 3-fold	*G. rostochiensis*	Cronin *et al*., 1997

use them as a source of nutrients, or such evidence is lacking. We have discussed antagonistic bacteria according to their mechanism of action.

10.2.1 Production of enzymes, toxins and antibiotics

In some cases antagonistic bacteria are able to produce enzymes such as proteases, collagenases and lipases that act as pathogenicity and virulence factors against free-living and plant-parasitic nematodes (Table 10.1). *Bacillus nematocida* strain B16 produces an alkaline serine protease that degrades the cuticle of *P. redivivus* juveniles. When crude extract (100%) of purified enzyme was used, it caused 95% death after an exposure of 48 h (Niu *et al*., 2006a). This bacterial species also produces the neutral protease Bae16 and the extracellular serine protease Bace16 that also have cuticle-degrading activities against *P. redivivus* and the plant-parasitic nematode (PPN) *Bursaphelenchus xylophilus* (Niu *et al*., 2006b, 2007). The extracellular alkaline protease BLG4 from *Brevibacillus laterosporus* strain G4 (BLG4) degrades 95% of the epidermis of *P. redivivus* following an exposure of 60 h (Huang *et al*., 2005). The neutral protease NPE-4 is also produced by *B. laterosporus* strain G4 and it acts synergistically with BLG4 in the degradation of the cuticle and the epidermis of nematodes (Huang *et al*., 2005; Tian *et al*., 2006, 2007). Spores of *B. laterosporus* attach to nematode's cuticle, but the mechanisms of attachment and germination have not been studied yet (Tian *et al*., 2007). Cloning and sequencing of the gene coding for the enzymes produced by *B. nematocida* B16 and *B. laterosporus* G4 have shown that they are highly conserved between these bacteria (Niu *et al*., 2006a; Tian *et al*., 2006).

Stenotrophomonas maltophilia strain G2 has high nematicidal activity against *B. xylophilus* and *P. redivivus* and its serine protease was found responsible for the pathogenicity factor involved in cuticle degradation (Huang *et al*., 2009). Chitinases produced by S. *maltophilia*, *Lysobacter enzymogenes* and *Bacillus* sp. strain D2 contribute to the degradation of the eggshell and inhibit hatching in *Globodera rostochiensis* and other nematodes, both under *in vitro* conditions and in soil (Cronin *et al*., 1999; Chen *et al*., 2006; Margino *et al*., 2012).

Species of the genus *Pseudomonas* produce a series of compounds with activity against nematodes. The extracellular protease aprA from *Pseudomonas fluorescens* strain CHA0 causes death and inhibits egg hatching in *Meloidogyne incognita* (Siddiqui *et al*., 2005). A mutation in the global regulator gene *Gac*A, which controls the expression of *apr*A, decreases the activity of the bacterium against *M. incognita*. Hydrogen cyanide and 2-4-diacethylphloroglucinol produced by strain CHA0 also play a role in nematode control (Siddiqui and Shaukat, 2003). The antibiotic 2,4-diacetylphloroglucinol produced by strain F113 of *P. fluorescens* is responsible for the ability of this strain to double the egg hatching in *G. rostochiensis* and also diminish three-fold the mobility of the hatched juveniles *in vitro* and in soil. Mutants unable to produce the antibiotics were not as efficient as the wild-type strain (Cronin *et al*., 1999).

Bacillus thuringiensis is considered a successful biocontrol agent in the management of insect pests (Bravo *et al*., 2011). After sporulation, *B. thuringiensis* produces the Cry (crystal) protein that has insecticidal and nematicidal properties. There are more than 300 *cry* genes of *B. thuringiensis* encoding Cry proteins that are classified into 68 families according to their sequence similarity (Crickmore *et al*., 1998; http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt). Among these families, Cry5, Cry6, Cry12, Cry13, Cry14, Cry21 and Cry55 show toxicity against nematodes (Guo *et al*., 2008), but only Cry5B, Cry5Ba2, Cry6A, Cry6Aa2 and Cry55Aa1 have been found active against PPNs (Guo *et al*., 2008; Luo *et al*., 2013). These proteins when ingested by nematodes are cleaved by the intestine proteases and the products cause the lysis of epithelial cells, ultimately resulting in death of the target pest. Cry6A with 54 kDa is among the proteins in the size range that may be ingested by *M. incognita*. Transgenic tomato plants expressing Cry6A reduced four-fold the reproduction of *M. incognita* (Li *et al*., 2007). Similar results were obtained by expressing an ingestible truncated fragment of the protein Cry5B in tomato roots (Li *et al*., 2008). Cry5Ba2, Cry6Aa2 and CryrAa1 showed toxicity to *Meloidogyne hapla* juveniles (Guo *et al*., 2008). The Cry proteins

of *B. thuringiensis* also produce an array of other virulence factors such as Cyt (cytolytic) inclusions, low molecular mass exotoxins, serine proteases, chitinases, collagenases and a metalloproteinase (Guttmann and Ellar, 2000; Raymond *et al*., 2010; Jisha *et al*., 2013; Luo *et al*., 2013). These toxins may act in concert with the Cry proteins enhancing the toxicity against insects and free-living nematodes (Raymond *et al*., 2010; Luo *et al*., 2013). There is a lack of information related to the activity of these virulence factors against PPNs. One of the reasons for the limited number of studies on PPNs with Cry proteins seems to be the size restriction imposed by the stylet, which does not allow most Cry proteins to be ingested. The favourite approach to nematode control with Cry proteins is transgenesis, and most transformed plants are subject to patenting by biotechnology companies.

10.2.2 Production of volatile compounds

Volatile compounds are classified into organic or inorganic and may be produced by bacteria. Bacteria of the genus *Xenorhabdus* are carried in the intestine of the entomopathogenic nematode *Steinernema*, whereas *Heterorhabditis* carries the bacterial genus *Photorhabdus* (Askary, 2010). These bacteria produce toxins that increase the virulence of the nematode carrier toward insects (Aatif *et al*., 2012). When these bacteria were tested against PPNs, they were shown to inhibit egg hatch and promote the mortality of *Meloidogyne* juveniles by allelopathy through the production of ammonia (Grewal *et al*., 1999).

Volatile organic compounds (VOCs) released by bacteria have a low boiling point, lipophilic nature and are in the liquid state only under high vapour pressure (Baldwin *et al*., 2006; Asensio *et al*., 2008; Heil and Ton, 2008). Production of VOCs is influenced by environmental conditions, availability and source of nutrients, oxygen, moisture and the microbial community (Leff and Fierer, 2008; McNeal and Herbert, 2009; Insam and Seewald, 2010). Many bacteria produce VOCs that exert some influence on nematodes (Table 10.1). Some of these VOCs have detrimental effects on nematodes, causing immobility, repellence or death, while others may attract them. In the database of volatiles emitted by microorganisms (DOVE-MO), there are 671 VOCs produced by 212 bacterial species (Effmert *et al*., 2012). VOCs produced by bacteria have some antagonistic effects against nematodes (Table 10.1). Gu *et al*. (2007) conducted *in vitro* screening of VOCs obtained from 200 bacterial isolates belonging to seven species. Among the 22 isolates which showed 100% nematicidal activity against *P. redivivus* and *B. xylophilus*, 21 belonged to three species of the genus *Bacillus* (*B. simplex*, *B. subtilis* and *B. weihenstephanensis*) and one was identified as *Serratia marcescens*. The nine VOCs that inhibited 100% of both nematodes were 2-octanol, benzeneacetaldehyde, decanal, benzaldehyde, 2-nonanone, 2-undecanone, cyclohexene, dimetyl disulfide and phenol. Benzaldehyde produced by all bacterial isolates showed higher nematicidal activity. *Bacillus megaterium* YFM3.25 and *Lysinibacillus mangiferahumi* M-GX18T produced a series of VOCs that killed *M. incognita* juveniles and inhibited egg hatching (Huang *et al*., 2010; Yang *et al*., 2012).

Some VOCs may be classified as virulence factors, allowing saprophytic bacteria to exploit nematodes as a source of nutrients. It has been shown that *B. nematocida* B16 produces at least seven VOCs that attract *C. elegans* toward their colonies and once the bacteria enter the nematode gut they secrete two proteases, Bace16 and Bae16 (mentioned above), with a broad substrate range that target primarily vital intestine proteins causing the host's death (Niu *et al*., 2010). This mechanism of attraction and subsequent killing of nematodes has not been shown for PPNs, but it provides a framework for future investigations and application in biocontrol. VOCs may also act as signaling molecules in the communication of bacterial cells in a density-dependent manner, a coordinated behaviour to regulate the expression of genes known as quorum sensing. Among these infochemicals, homoserine lactones are commonly found. *Pseudomonas aeruginosa* produces a homoserine lactone that attract the nematode *C. elegans* (Beale *et al*., 2006), but whether this effect is similar for phytonematodes needs investigation. Several species of *Streptomyces* and *Bacillus*

produce dimethyl disulfide, which shows inhibitory activity against nematodes and also induces systemic resistance in plants (Schöller *et al*., 2002; Faruk *et al*., 2010; Huang *et al*., 2012). Additionally, VOCs produced by *B. subtilis*, *Bacillus amyloliquefaciens* and *Enterobacter cloacae* promote growth of model weed plant *Arabidopsis thaliana* (Ryu *et al*., 2003). Thus, VOCs play diverse roles in plant–nematode interactions and may be used for developing new applications in the integrated management of PPNs.

10.3 Parasitic Bacteria

10.3.1 *Pasteuria*

Pasteuria species are the best studied among the nematode-parasitic bacteria. Despite the unquestionable potential of *Pasteuria* spp. as biocontrol agents, the narrow host range and the difficulties of mass production are the main constraints for its commercial development. However, a company known as *Pasteuria* bioscience, now a branch of Syngenta claims to produce cost-effective spores for field applications. Little information is available about the molecular mechanisms of *Pasteuria* virulence to nematodes, mainly because the technology for *in vitro* culturing is not yet widespread.

The attachment of *Pasteuria* spores occurs by a mechanism called 'velcro-like' (Davies, 2009), wherein collagen-like fibres on the spore surface and mucins on the nematode surface interact. The composition of these surface fibres determines the host range of particular *Pasteuria* isolates. The attachment itself may be considered as a pathogenicity mechanism against *Meloidogyne* species once juveniles with more than four endospores encumbered have their infectivity diminished (Davies *et al*., 1988; Kariuki *et al*., 2006). The life cycle of these bacteria shows considerable diversity. Spores of *Pasteuria penetrans* and *Pasteuria nishizawae*, parasitic to *Meloidogyne* and cyst nematode species, respectively, germinate only after the nematode starts feeding and spores are produced inside adult females (Sayre and Starr, 1985; Sayre *et al*., 1991). This mode of parasitism has been referred to as synchronized with the host's life cycle where the bacteria exploits the supply of nutrients from its nematode host to produce the maximum number of spores (Sturhan *et al*., 1994). In this case the bacteria do not kill the host immediately, but act by castrating it or diminishing the amount of eggs produced. Although not much information is available about the molecular mechanisms of virulence, bacterial colonies possibly secrete a series of effector molecules that act by lowering the nematode defences to allow the development of the parasite in the pseudocoelom. On the other hand, spores of *Pasteuria* sp. parasitic to *Heterodera avenae*, *Heterodera goettingiana* and *Tylenchulus semipenetrans* germinate and complete their entire life cycle in second-stage juveniles (J_2) (Fattah *et al*., 1989; Davies *et al*., 1990; Sturhan *et al*., 1994). Other *Pasteuria* isolates parasitize and produce spores in different stages of *Pratylenchus* spp., *Trophonema okamotoi*, *Helicotylenchus lobus* and other vermiform nematodes (Starr and Sayre, 1988; Ciancio *et al*., 1992; Inserra *et al*., 1992). These isolates have no synchronization with the host's life cycle and kill the nematode before it reaches maturity, leading to the production of fewer propagules when compared to isolates from *Meloidogyne* and some cyst nematodes. This diversity in life cycle implies that these bacteria are composed of species with different virulence mechanisms. Parasitized nematode specimens with mature spores inside the pseudocoelom retain their movement (Fattah *et al*., 1989), as the biotrophic lifestyle of the bacterium seems to lack toxins to kill the host and enzymes to degrade the cuticle. Parasites with moderate virulence towards their hosts were shown to have more chances of propagating themselves than highly virulent ones (Jensen *et al*., 2006). Probably, isolates with higher virulence will be eliminated together with the host, but all *Pasteuria* isolates reported to date appear to be in equilibrium with their hosts (Ciancio, 1995; Atibalentja *et al*., 1998). Intermediate levels of virulence and genetic diversity are among the factors that control the long-term maintenance of host–parasite relationships (Jensen *et al*., 2006). Both factors appear to contribute to the ecological and evolutionary success of *Pasteuria*.

10.4 Symbiotic Bacteria

Intracellular symbionts are found in association with PPNs in some occasions. Three different species of the newly described and yet uncultivable genus *Candidatus* Xiphinematobacter that belongs to the phylum Verrucomicrobia were encountered inside the ovaries of three species of the *Xiphinema americanum* group that reproduce by thelytokous (mother-to-daughter) parthenogenesis (Vandekerckhove *et al*., 2000). Since other species of *Xiphinema* are free of the symbionts, it has been postulated that the bacteria induce *Xiphinema* to reproduce by thelytokous parthenogenesis, manipulating the host's reproduction to assure its vertical transmission in a way similar to the strategy adopted by *Wolbachia* in arthropods (Saridaki and Bourtzis, 2010).

Wolbachia (phylum Proteobacteria, family Rickettsiaceae) is reported to colonize the ovaries of *Radopholus similis* and *Radopholus arabocoffeae*. The bacterium was classified in the new, so called supergroup I of *Wolbachia* (Haegeman *et al*., 2009). It is only known from *Radopholus* among the PPNs. Although its role could not be determined, the infection rate was high (Haegeman *et al*., 2009). It is not known whether other populations and species of *Radopholus* harbour the bacteria because an extensive search has not been attempted.

The bacterium *Candidatus* Paenicardinium endonii classified in the phylum Bacteroidetes was found in males, females and juveniles of the soybean cyst nematode, *Heterodera glycines*. Other populations of this species and other cyst nematodes did not carry *Cand.* P. endonii. The morphological characteristics of the bacterium are identical to endosymbionts described for *H. glycines*, *H. goettingiana* and *G. rostochiensis* (Noel and Atibalentja, 2006). The bacterium does not seem to be causing any disease and its occasional occurrence indicates that it is not essential for the ecological fitness of the host, whereas *Cand*. Xiphinematobacter and *Wolbachia* behave as reproductive parasites in female nematodes, possibly manipulating the host's reproductive behaviour. *Cand*. Paenicardinium occurs in ovaries and other parts of the cyst nematode with no evidence of reproductive manipulation (Vandekerckhove *et al*., 2000; Noel and Atibalentja, 2006; Haegeman *et al*., 2009).

10.5 Methods to Study Pathogenicity Mechanisms

Several bacterial genes encoding pathogenicity and virulence factors have been sequenced. This information may be used to develop PCR screening assays to recover homologous sequences from different bacteria. The availability and ease to obtain bacterial genome sequences in the present day allows the use of this information to perform comparative analysis of genomes to identify pathogenicity and virulence factors from bacteria against PPNs. Random and site-directed mutagenesis followed by complementation is one of the most employed methods to prove that a gene is a pathogenicity factor. This method provides unequivocal evidence in most cases, but care must be taken when pathogenicity factors belong to gene families.

Studies on pathogenicity genes that are induced in the nematode host may be done by *in vitro* expression technology (IVET) or recombinase (R-IVET). These techniques allow the identification of genes expressed in the interaction with the host nematode as compared to normal *in vitro* conditions (Angelichio and Camilli, 2002; Castillo *et al*., 2008) and can easily be applied for the antagonistic bacteria with a free-living nematode as a host model. Other techniques, such as RFLP-differential display and subtractive hybridization make possible the detection of genes differentially expressed when bacteria are associated with the host nematode. However, RNA sequencing has the potential to replace these techniques because it has the advantage of the reduced cost of mass sequencing coupled with bioinformatics. RNA sequencing has also been helpful in replacing array technologies to study innumerous genes in parallel (Riccombeni and Butler, 2012). The results of mass sequencing need confirmation by techniques such as quantitative real time PCR (qPCR). PPNs are not amenable to transformation, but blocking gene expression by RNA silencing has been developed and may be used in pathogenicity studies (Urwin *et al*., 2002).

Microscopy-based methods combined with immunological techniques allow the localization of tagged proteins or GFP-marked bacteria in the body of nematode hosts. This allows the detection of protease from *B. nematocida* isolated from the intestine of *C. elegans* (Niu *et al*., 2010).

Purification of proteins followed by their identification with mass spectrometry (MS) can also be performed when the required facilities are available. Sequencing the genes encoding the purified proteins and subsequently obtaining a mutant phenotype is known as reverse genetics, as opposed to sequencing a gene from a mutant phenotype. Reverse genetics have been used to obtain the genes coding an extracellular protease that acts as a pathogenicity factor of *B. laterosporus* G4 against nematodes (Huang *et al*., 2005). Volatile organic compounds may be identified by gas chromatography/MS (GC/MS) analysis. Other techniques to study VOCs include solid phase microextraction (SPME), proton transfer reaction/MS (PTR-MS) that allows online VOC measurements and also may be combined with time-of-flight (TOF) or gas chromatography, selected ion flow tube/MS (SIFT-MS) and secondary electron spray ionization/MS (SESI-MS) and analytical chemistry (Wenke *et al*., 2012).

10.6 Outlook

Bacteria produce several proteins and metabolites that act as pathogenicity and virulence factors, but unfortunately there is a lack of sufficient knowledge about the role of these compounds against PPNs. Most studies are done with model nematodes, such as *P. redivivus* and *C. elegans* where the primary interest is the study of animal pathogens. There is no question about the importance of these model nematodes to study pathogenicity, but in many instances extrapolations cannot be done. For example, when *C. elegans* feed on nematotoxic bacteria, such as *B. nematocida*, *Xenorhabdus* spp., *Photorhabdus* spp. or Cry proteins (Grewal *et al*., 1999; Niu *et al*., 2010; Luo *et al*., 2013) they are readily killed, but the stylet of plant parasites protect them from the same fate.

A considerable part of the evidence about the role of some virulence factors against nematodes is merely circumstantial. The mutants unable to produce these factors have not been studied. Another aspect that complicates studies of pathogenicity and virulence factors is that they rarely act alone. Pathogenicity and virulence factors frequently act synergistically to produce the phenotypes observed. Mutations to inactivate pathogenicity and virulence factors may prove unsuccessful because many of them belong to gene families, so deletion of one gene is compensated by another gene of the family.

Though extensively studied, the virulence mechanisms that *Pasteuria* spp. utilize to develop are apparently undetected in the host's pseudocoelom and remain a mystery. No bacterial effectors have so far been found to regulate the host's defence to facilitate bacterial development. The genome of *Pasteuria* will probably allow extensive comparative genomics and the identification of these effectors. Phylogenetic analysis revealed that *Pasteuria* is closely related to non-parasitic bacteria of the genus *Bacillus*. Comparisons performed with several bacilli genomes and the unfinished *Pasteuria* sequences revealed similarities between these bacteria and helped to understand the sporulation process, resulting in improved media to grow these bacteria *in vitro* together with supernatants of helper bacteria (Dupponois *et al*., 1999; Gerber and White, 2001; Hewlett *et al*., 2004). The nature of the supernatants produced by the helper bacteria is still unknown. Therefore, the challenge remaining is the manipulation of the spore attachment process to make the bacterial strains effective for a broader host range.

Other than the identity of the intracellular symbionts in PPNs, virtually nothing is known about them. Are they symbionts or obligate nematode parasites? Which effectors are they producing to lower the defences of the nematode host? Are they producing essential molecules such as glutathione in exchange for other nutrients and protection as in filarial nematodes? Are they really manipulating the host's sexual behaviour? Further research should also focus on the distribution of symbiotic bacteria in other populations of the hosts from which they have been reported

and also in other genera of phytonematodes of diverse geographical regions.

Studies on mechanisms of bacterial pathogenicity to PPNs are still in their initial stage and most of the limited knowledge available is on antagonistic bacteria, but the technology available seems sufficient to study the obligate parasites. Now it is up to the dedicated research workers to undertake this exciting task of bacterial virulence mystery.

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11 Nematophagous Bacteria: Survival Biology

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11.1 Introduction

Plant-parasitic nematodes play an important role among the pathogens that cause serious damage directly to the plants and indirectly to the growers. Their destructive action on the root system or aerial part affects the absorption and translocation of nutrients to the plant, altering its physiology and predisposing it to other complex diseases and environmental stresses (Paula *et al*., 2011). The great majority of plant-parasitic nematodes pass at least part of their life cycle in the soil, and their activity is influenced by the variation of physical (temperature, humidity and aeration), chemical (defensives and fertilizers) and physiological (Alves and Campos, 2003; Ferraz *et al*., 2010) factors. The biological component of the soil ecosystem is mainly important in limiting or stabilizing nematode populations by means of competition, parasitism, predation and production of toxic compounds. Use of natural enemies in biological control is being studied widely (Ferraz and Valle, 1997) and it appears to be an ecologically sustainable alternative in the management of phytonematodes, because it produces less damage to the environment compared to conventional methods (Coimbra and Campos, 2005). Various organisms are considered natural enemies of phytonematodes, such as tardigrades, viruses, arthropods, nematode predators, acari, fungi and bacteria (Stirling, 1991; Alves and Campos, 2003). Despite diversity among antagonists, not all these organisms present the potential for being used in practical biological control of nematodes, except some bacteria and fungi which are considered the most probable candidates and can also be used as commercial products (Stirling, 1991; Freitas *et al*., 2001). A better understanding of biology and survival of bacteria can provide a basis for human intervention and optimizing their pathogenic activity against phytonematodes (Siddiqui *et al*., 2003). Several factors influence the survival of bacterial populations in the soil. These may be the intrinsic physiological characteristics and soil abiotic and biotic factors (Van Elsas and Heijnen, 1990; Van Veen *et al*., 1997). The present article is focused upon the factors that influence the survival of nematophagous bacteria in soil. The effect of these factors upon the pathogenic potential of bacteria has also been discussed.

Bacteria are cosmopolite organisms that in their constant association with the rhizosphere may be antagonistic to phytonematodes

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(Freitas and Carneiro, 2000). For example, *Pasteuria penetrans* (Thorne) Sayre and Starr are parasites of phytonematodes, while other bacteria promote a reduction in the nematode population through other modes of action. Thus, bacteria may be categorized into two groups: (i) nematode parasitic (*Pasteuria* spp.); and (ii) nematode non-parasitic (rhizobacteria, endophytic bacteria, entomobacteria and actinomycetes) (Schroth and Hancock, 1982; Stirling, 1991; Meyer, 2003). The non-parasitic bacteria that have been studied for biocontrol of phytonematodes include species from the genera *Acidovorax*, *Agrobacterium*, *Alcaligenes*, *Arthrobacter*, *Aureobacterium*, *Azotobacter*, *Beijerinckia*, *Breuundinzonas*, *Brevibacillus*, *Burkholderia*, *Chromobacterium*, *Citrobacter*, *Clavibacter*, *Clostridium*, *Comamonas*, *Corynebacterium*, *Curtobacterium*, *Desulfovibrio*, *Enterobacter*, *Flavobacterium*, *Gluconobacter*, *Hydrogenophaga*, *Klebsiella*, *Methylobacterium*, *Phyllobacterium*, *Rhizobium*, *Serratia*, *Stenotrotrophomonas*, *Streptomyces*, *Pseudomonas* and *Bacillus*, most notably the last two (Jacq and Fortuner, 1979; Kloepper *et al*., 1991, 1992; Stirling, 1991; Racke and Sikora, 1992; Guo *et al*., 1996; Cronin *et al*., 1997; Hallmann *et al*., 1997, 2002; Duponnois *et al*., 1999; Neipp and Becker, 1999; Siddiqui and Mahmood, 1999, 2001; Jonathan *et al*., 2000; Tian and Riggs, 2000; Tian *et al*., 2000; Mahdy *et al*., 2001; Meyer *et al*., 2001; Insunza *et al*., 2002; Mena and Pimentel, 2002; Meyer, 2003; Aravind *et al*., 2010).

11.2 Parasitic Bacteria – Mode of Action and Survival

Pasteuria penetrans presents two distinct modes of action in the biological control of *Meloidogyne* spp.: (i) preventing females from reproducing by parasitizing them; and (ii) reducing the infectivity of second-stage juveniles (J_2) by adhering endospores on their cuticles (Alves *et al*., 2002, 2004). The J_2 of root-knot nematode moves in the soil to search for the root of a host plant on which to feed; it comes into contact with endospores of *P. penetrans*, which are highly adhesive, that fix firmly on the nematode's cuticle thus encumbering dozens of endospores on the body of an individual J_2 (Alves *et al*., 2002). Soon after that germination of endospores takes place. The germination tube of the endospore emerges from inside the body of the nematode, reaching the pseudocoelom. The branch formation takes place at the tip of the germinating tube, forming a septate mycelium that produces vegetative colonies, and these divide into new colonies. During the process of development, fragmentation of colonies, thickening of terminal cells and production of endospores take place. The cuticle of the infected nematode decomposes and the endospores are released into the soil and re-start the cycle by infesting other J_2 (Stirling, 1984; Brown *et al*., 1985). When the nematodes become unable to penetrate the root any more, it is considered that the soil has become suppressive and the nematode population tends to fall dramatically (Freitas and Carneiro, 2000).

Pasteuria penetrans is one of the most promising biocontrol agents (BCAs) of phytonematodes due to its various desirable characteristics, which include resistance to desiccation and temperature oscillations, survival for longer periods in the absence of host, absence of natural enemies, higher longevity of stored endospores without losing viability, compatibility with pesticides, fertilizers and other BCAs, non-toxicity to humans and animals and its ease of use in conjugation with common crop management practices (Stirling and Wachtel, 1980; Brown and Nordmeyer, 1985; Mani, 1988; Williams *et al*., 1989; Oostendorp *et al*., 1990; Tzortzakakis and Gowen, 1994; Español *et al*., 1997; Sosamma and Koshy, 1997; Mukhtar and Ahmad, 2000; Rao *et al*., 2000; Alves *et al*., 2011). The survival of *P. penetrans* is influenced by abiotic factors especially the properties of soil, temperature and humidity, but most studies have been carried out under controlled conditions and therefore it is necessary to conduct studies in natural environments to take into account the interdependence of complex soil factors (Mateille *et al*., 2009).

11.2.1 Factors affecting survival of parasitic bacteria

Soil texture and organic matter content

Spatial diversity and localization of habitat pores in the soil are the two important factors for accessibility and, consequently, for the survival

of bacteria (Postma and Van Veen, 1990). Factors related to the soil may affect adhesion (Alves *et al*., 2002), development and survival of *P. penetrans* (Davies *et al*., 1991). Knowledge about survival and activity of the spores of *P. penetrans* in the soil is essential in predicting the success as a BCA. Dabire and Mateille (2004) investigated the influence of different soil textures (sandy, sandy-clay and clay) on the spread and development of *P. penetrans* in cultivated soils and the effect of clay content on spread and retention of spores in the soil. It was noted that 53% of the spores were leached by the flow of water in sandy soil, 14% in sandy-clay soil and only 0.1% in clay soil. On the other hand, in intensely irrigated clay soils (29% clay) there was a reduction in the vertical movement of spores. Dabiré *et al*. (2007) compared different combinations of bare soils containing from 1.1 to 57% clay, and observed that the best balance between leaching and retention of spores occurred in soils containing 10–30% clay. Costa *et al*. (1998) investigated factors that influence the survival of *P. penetrans* in soils in Sri Lanka and observed that past cultivation and drainage of the soil had an effect on the prevalence of the bacterium. Among the areas with occurrence of *P. penetrans*, 87% were well drained. In 74% of the samples from areas cultivated for more than 30 years, the presence of *P. penetrans* was detected. On the other hand, presence of the bacterium was only noted in 9% of the areas cultivated for less than 15 years. Trudgill *et al*. (2000) studied the occurrence of *P. penetrans* in fields cultivated with vegetables in some countries and correlated the frequency of the bacterium with the soil type. The frequency of *P. penetrans* was lower in sandy soils of Senegal, while in Ecuador the occurrence was independent of soil characteristics. In Burkina Faso the occurrence of the bacterium decreased in soils with higher clay and organic matter content. Spaull (1984) reported that *P. penetrans* occurred more frequently in sandy and sandy-clay soil than in clay-sand and clay soils. In contrast, Verdejo-Lucas (1992) found *P. penetrans* more abundant in clay-sand than in sandy clay in kiwi orchards.

As observed, in some cases there was a greater possibility of *P. penetrans* occurring in soils with higher sand content, while in other cases, clay soils were more favourable. This demonstrates that under natural conditions there are a number of other factors in the soil that are inter-related and that work concomitantly, and may play a decisive role in the occurrence and survival of *P. penetrans*. For multiplication and survival of *P. penetrans* in the field, it is necessary to keep in mind that adding high organic matter content to the soil can cause an increase in the phenol concentrations in the roots, which is deleterious to the giant cells on which nematodes feed. As the reproduction of *P. penetrans* depends on the presence of nematodes, any factor that negatively affects these pathogens, such as the presence of organic matter, will consequently affect the bacterium (Alves *et al*., 2007).

Soil temperature

Pasteuria penetrans is a mesophyllic bacterium. Freitas and Carneiro (2000) reported that its resistance to heat is variable, because heating affects its adhesion and infectivity differently, depending upon the length of time when the endospores or vegetative structures are exposed to high temperatures. Mani (1988) demonstrated that endospores of *P. penetrans* remained viable for more than 1 year when stored at 10–30°C or at ambient temperature (18.5–36°C); beyond this period, the bacterium was still found able to multiply on *Tylenchulus semipenetrans*. On the other hand, average annual temperatures lower than 10°C may affect the survival of *P. penetrans*, as observed in Hawaii (Ko *et al*., 1995). According to Giannakou *et al*. (1997), knowledge about the survival of endospores of *P. penetrans* at temperatures that are lethal to *Meloidogyne* spp., such as those obtained during soil solarization, may prove helpful in finding out a practical value in thermic treatments for control of phytonematodes. Freitas *et al*. (2000a) verified that the phases of the life cycle of *P. penetrans* were delayed or eliminated due to exposure to temperatures above 50°C obtained with solarization for 10 days. Incorporating cabbage in the soil and incubating at 50°C for 10 days affected formation of endospores in females of *Meloidogyne arenaria*. The gases that could be toxic to the bacterium are liberated during decomposition of cabbage. Growers can boost

reproduction and survival of *P. penetrans* if they apply it in agricultural areas where temperatures over 20°C predominate, with 35°C being the optimum temperature for the bacterium to grow (Hatz and Dickson, 1992).

Storage conditions

The common method to store endospores of *P. penetrans* is to dry the roots containing females of *Meloidogyne* spp. infected with the bacterium. These roots can be stored intact or may be milled. The milling has no adverse effect on the survival of *P. penetrans* (Stirling and Wachtel, 1980). Endospores can also be stored in a suspension of distilled or tap water and refrigerated at 4°C for several months. They can be frozen and after thawing they remain viable and capable of adhering to juvenile root-knot nematodes (Hewlett and Serracin, 1996). Strains of *P. penetrans* have been found to survive for 6 weeks in dry, moist and wet soils, and in a soil with fluctuating humidity, without losing their capacity to adhere to the nematode (Oostendorp *et al*., 1990). According to Hewlett and Serracin (1996), endospores of *P. penetrans* present in air-dried soil maintained in ambient temperature in a hermetically sealed receptacle survived for many years. Español *et al*. (1997) reported that endospores of *Pasteuria* sp. stored for up to 6 years in dry roots adhered to the nematodes, although there was no infection. In another study, the storage of *P. penetrans* for 11 years at ambient temperature in the form of dry root powder did not reduce the capacity of the bacterium to adhere to J_2 of *Meloidogyne javanica*, but significantly reduced the pathogenic ability of bacteria (Giannakou *et al*., 1997).

A number of studies on the use of *P. penetrans* in the biocontrol of phytonematodes have been carried out (Mankau, 1975; Singh and Dhawan, 1996). Several researchers have also reported the use of *P. penetrans* in association with other antagonists and/or other nematode management practices, such as mycorrhizal fungi (Rao *et al*., 2000), antagonist plants (Ferraz and Valle, 1997), non-parasitic bacteria (Duponnois *et al*., 1999), nematicides (Brown and Nordmeyer, 1985), plant extracts (Mukhtar and Ahmad, 2000), fungicides (Melki *et al*., 1998) and soil solarization (Tzortzakakis and Gowen, 1994). These associations between *P. penetrans* and other management practices in the management of phytonematodes apparently do not have any negative effect on the survival of the bacterium. Endospores of *P. penetrans* can also survive sonication (Williams *et al*., 1989), a procedure used to rupture the bacterial sporangium wall and increase the capacity of the endospores to adhere to the nematode (Stirling *et al*., 1986). Other factors affecting the pathogenicity of *P. penetrans* are ammonium nitrate (Chen and Dickson, 1997) and soil-fumigant nematicides (Freitas *et al*., 2000b).

11.3 Non-parasitic Bacteria – Mode of Action and Survival

Few studies have been conducted to prove the mode of action of rhizobacteria on phytonematodes. Rhizobacteria act on the following.

- Nematode eggs, causing cell death and interrupting embryonic development of juveniles due to production of nematotoxic substances.
- Eclosion of J_2, so that hatched J_2 have a need to receive stimuli from root exudates. The rhizobacteria, probably on degrading these exudates, interfere negatively in eclosion. It is also reported that J_2 inside the eggs are inactivated or deformed, losing their ability to hatch.
- Initiation of mobility, i.e. directing the juveniles towards the root: the rhizobacteria alter the composition of the root exudate and the nematodes no longer recognize the chemotropic stimulus that directs them to the roots; the bacteria may also produce nematostatic substances that reduce the nematodes' mobility and prevent them from reaching the roots.
- Host recognition: substances produced by the rhizobacteria are absorbed by the roots and may alter their chemical composition, so that the nematodes no longer recognize their host. In addition, the agglomeration of the bacteria on the root surface may interfere in the nematode's recognition of the host plant.
- Penetration of the nematodes into the root: toxic or repellent substances produced

by rhizobacteria and concentrated in the rhizoplane can inhibit penetration of nematodes into the host root.

- Feeding: the hypersensitivity reaction is an important mechanism of plant resistance to phytonematodes. The products of rhizobacterial metabolism can be absorbed by the plant and trigger this type of reaction in the host tissues, making it difficult or impossible to establish a feeding site for endoparasitic nematodes. Furthermore, by absorbing rhizobacterial metabolites, the plants may trigger systemic resistance to phytonematodes.
- Reproduction: females of sedentary endoparasitic nematodes, such as *Meloidogyne* spp. and *Heterodera* spp., need to be in contact with their feeding sites for obtaining their food adequately. Bacterial metabolites have the capacity to induce alterations in the form, size or content of these sites, and this causes poor feeding among females, jeopardizing their reproduction (Becker *et al*., 1988; Oostendorp and Sikora, 1990; Ferraz *et al*., 2010).

It is possible that a certain rhizobacterial isolate will exhibit not just one mode of action on the nematode, but rather a set of events that result in its effectiveness overall.

Both biotic and abiotic factors affect the survival of non-parasitic bacteria.

11.3.1 Biotic factors

Physiological characteristics of the bacterium

The efficacy of bacteria that are antagonist to phytonematodes depends on the physiological state of their populations (Stark and Firestone, 1995). Bacteria introduced in the soil should possess survival ability that is adaptable to abiotic and biotic stress in the environments (Yu-Huan and Mazzola, 2001). The conditions required for culturing rhizobacteria can have an impact on the production of antimicrobial metabolites (Duffy and DeAfago, 1999), viability during the storage period as well as even on antagonistic efficiency against phytonematodes (Slininger *et al*., 1998). Long-term storage and repeated cultivation of these BCAs may result in physiological alterations (Persson *et al*., 1990) and reduction in survival ability of BCA in natural soils (Weller, 1988).

Yu-Huan and Mazzola (2001) reported that *in vitro* exposure of fluorescent *Pseudomonas* to conditions that induce resistance to common stresses in the soil may bring about improvements in the colonization of roots and in the efficiency of biocontrol. As demonstrated in experiments carried out *in vitro*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Escherichia coli* may develop self-protection against stress factors such as ethanol, moderate heat and osmotic and oxidative stress (Jenkins *et al*., 1988, 1990; Givskov *et al*., 1994a,b). Despite these results, such information needs to be validated in field experiments. Although they are not well understood, physiological characteristics can influence the activity and survival of bacteria in the soil, but it is important to note that different species behave in different ways (Van Veen *et al*., 1997). For example, in cultivated or uncultivated soil, *Arthrobacter* sp. survived for a longer time and in greater numbers than *Flavobacterium* (Thompson, 1980).

The first stage in using bacterial strains efficient in biocontrol of phytonematodes involves a thorough selection (Van Veen *et al*., 1997). Liljeroth *et al*. (1991) demonstrated that bacteria preferring the tip of wheat roots were physiologically different from those present in the older parts of the roots. Microbial cells may survive in biofilm on the root surface, the phenomenon known as quorum sensing, which is characterized by an intra- and interspecies communication system in microorganisms, based on the emission of stimuli and responses dependent on population density. This type of interaction reflects the behaviour of microorganisms, demonstrating their capacity to inhabit diverse environments, receive information from their medium, communicating with different species, monitoring their population density and principally, regulating their gene expression, controlling cell processes such as sporulation, formation of biofilms, expression of virulence factors, production of bacteriocines and antibiotics, and survival (Wisniewski-Dyé and Downie, 2002; Daniels *et al*., 2004). Another strategy for improving the efficacy and survival of rhizobacterial strains is the use of genetically modified microorganisms, as well as the expression of

heterologous genes (Drahos *et al*., 1988). Several authors have studied the survival of strains of genetically modified *P. fluorescens* (Van Elsas *et al*., 1986; Van Overbeek *et al*., 1990). Nevertheless, sufficient studies on the impact of these bacteria on the environment are needed (Van Veen *et al*., 1997). Van Elsas and Van Overbeek (1993) have shown that there are genes in existence that may be activated by specific root exudates. A promoter identified in *P. fluorescens* was found specifically induced by the proline present in the rhizosphere of gramineous species (Van Overbeek and Van Elsas, 1995).

Understanding the response at a molecular level of bacterial populations to environmental stress in the soil can prove fundamental for researchers to predict and manipulate bacterial activities in the soil. It is important to emphasize upon differences between bacterial activity in the rhizosphere and outside the rhizosphere. However, to understand the effects of soil upon its microbial inhabitants, it is very important to obtain a more refined vision of the factors that control bacterial activities in specific sites in the soil, inside the soil aggregates or on the surface, in small pores of the soil and at different places (young and old regions of the root), in the soil of the rhizosphere or on the root surface (Van Veen *et al*., 1997). The physiological state of the individual microbial cell in a population in the soil is not likely to be uniform (Van Elsas and Van Overbeek, 1993). Bacterial populations in a soil can be affected in a more homogeneous way when drastic changes take place, such as drought, flooding, excessive cold or heat (Van Veen *et al*., 1997). There are various methods described to determine *in situ* physiology of the bacterial cells introduced in the soil (Bottomley, 1994; Van Veen *et al*., 1997).

Interactions with other organisms

With the introduction of nematophagous bacteria in soil, a competition starts between the indigenous populations of bacteria already present in soil and the introduced one for available substrate and biological space. This may affect the populations of nematophagous bacteria introduced in the soil (Postma *et al*., 1990; Alves *et al*., 2012). The establishment and survival of nematophagous bacteria is not always satisfactory, because after they have been introduced in the soil they become subject to soil microbiostasis (Ho and Ko, 1985). In fact, populations of nematode-antagonist bacteria, such as species of *Pseudomonas*, *Flavobacterium*, *Alcaligenes* and *Rhizobium* can dwindle after being introduced in the soil (Postma *et al*., 1988; Van Elsas and Van Overbeek, 1993; Cleyet-Marel *et al*., 1995). Molina *et al*. (2000) reported that *P. putida* KT2440 influenced the survival of natural bacterial populations in greenhouse and field conditions.

Protozoa play an important role as population regulators for bacteria in the soil. As demonstrated by various authors, there is a decline in bacterial populations when these are introduced in sterile soils together with predatory protozoa (Heijnen *et al*., 1988; Wright *et al*., 1995). Slow-growing bacteria show a decline in their populations in habitats where predators are present. However, these bacterial populations may be quickly regenerated if nutrients are made available by grazing protozoa (Sinclair and Alexander, 1989).

Successful colonization of roots by phytonematode-antagonist bacteria depends on their rhizospheric competence (Weller, 1988). Root colonization is a prerequisite for such bacteria to work efficiently in biological control (Van Veen *et al*., 1997). Kozdrój *et al*. (2004) found rhizosphere of maize plants favoured the survival of introduced cells of *Pseudomonas chlororaphis* IDV1 and *P. putida* RA2. According to Molina *et al*. (2000), *P. putida* KT2440 established itself in uncultivated soils 3 days after being introduced, without interfering in populations of various bacteria indigenous to the soil, including species of fluorescent *Pseudomonas*. The capacity of a rhizobacterium to use one or more of the many compounds produced by the plant roots may confer a competitive advantage over the indigenous population (Van Elsas *et al*., 1992).

11.3.2 Abiotic factors

Texture and pH of the soil

Van Elsas *et al*. (1986) introduced a strain of *P. fluorescens* in sandy-clay soil and clay-silt soil for 3 years and found the survival of

bacteria was best in the soil with a finer texture. Clay surfaces can provide protection for bacteria (Marshall, 1975). Foster (1988) demonstrated that after treating soil with chloroform, the microorganisms were found only in deposits of mucigel or in the inner parts of soil micropores. It was concluded that certain microhabitats of the soil were impenetrable to chloroform, acting as places of protection for the microorganisms. The survival of strains of *P. fluorescens* and *Bacillus subtilis* was studied in two soils with different textures, both planted with wheat. The population of *B. subtilis* decreased quickly in both soils and then stabilized. *P. fluorescens* showed a slow and constant decline in both soils. The survival of *P. fluorescens* was better in the clay soil than in the sandy soil (Van Elsas *et al*., 1986).

The introduction of montmorillonite and illite in a natural soil sample increased the density of indigenous populations of fluorescent *Pseudomonas* (Hopper *et al*., 1995). In other studies, it was noted that soils rich in clay also had a positive influence on survival of introduced lineages of fluorescent *Pseudomonas* (Van Elsas *et al*., 1986; Heijnen *et al*., 1992, 1993). The ability of *Pseudomonas* spp. to survive and colonize in different environments is due to the fact that bacterial cells adhere efficiently to soil particles (Duque *et al*., 1993). Furthermore, these species possess metabolic motility and versatility, which permits them to use various sources of carbon, nitrogen, sulfur and phosphorus (Chaudlhry and Chapalamadugu, 1991; Ramos *et al*., 1994).

To ensure their survival, it is important that the bacteria can find a protected place after they are introduced into the soil. Elliott *et al*. (1980) showed that interactions between nematodes, protozoa and bacteria are influenced by the distribution of porous spaces in soil. Postma *et al*. (1989) reported that soil should be irrigated before bacteria are introduced into it so that bacteria may protect themselves in small pores of soil. According to Hattori and Hattori (1976), soil particles and aggregates play an important role for the escape of these bacteria from antagonists. Wright *et al*. (1993, 1995) examined the action of the soil ciliate *Colpoda steinii* in the survival of *P. fluorescens* in soil containing pores of different diameters. The smaller pores (6 mm diameter) conferred greater protection for the bacterium against the protozoa than the pores with diameter varying from 6 to 30 mm. Another factor that may affect the survival of nematophagous bacteria is the pH of the soil. According to Van Veen *et al*. (1997), soil pH favours some microorganisms and hampers others, and also makes available nutrients such as phosphorus or aluminium. The use of soils with fine texture must be a priority, as they provide a better condition for the survival of antagonistic bacteria (Van Elsas *et al*., 1986).

Osmotic potential of the soil

Osmotic potential is one of the physical factors that influence proliferation and survival of nematophagous bacteria in a certain habitat. Osmoregulation can be defined as an active process carried out by organisms to adapt themselves to osmotic stress (Csonka, 1989). The cells can adapt to osmotic stress by means of accumulation of solutes in the cytoplasm after exposure to conditions of hyperosmolarity. Siddiqui *et al*. (2003) studied the impact of NaCl on the suppression of root-knot nematode *M. javanica* by bacteria *P. aeruginosa* IE-6S$^+$. The concentration of 0.8 M of NaCl was optimum for survival and *in vitro* growth of IE-6S$^+$, while nematicidal activity was greatest when the bacterium was exposed to 0.4 M of NaCl. The bacterium was highly sensitive to the high concentration (1.6 M) of NaCl. When IE-6S$^+$ was added to the soil, alone or with up to 0.8 M of NaCl, the efficacy of the bacterium against *M. javanica* improved. Egamberdiyeva (2007) reported that *Pseudomonas alcaligenes* PsA15 and *Bacillus polymyxa* BcP26 tolerated high temperatures and high salt concentrations, which gave them a competitive advantage for survival in arid and saline soils. Reina-Bueno *et al*. (2012) found high temperature not affecting the growth of *Rhizobium etli*, but the combination of high temperature and osmotic stress was deleterious to the bacterium.

Various aspects related to osmoregulation in bacteria have been studied, including interactions of biological macromolecules with solutes accumulated by the organisms in media with high osmolarity (Yancey *et al*., 1982), molecular biology of the accumulation

of cytoplasmic osmolytes (Le Rudulier *et al*., 1984) and models of osmoregulation related to genetic transcription (Higgins *et al*., 1987). Christian (1955a,b) observed that exogenous proline, an osmoprotector, reduced the inhibition of bacterial growth imposed by osmotic stress. Bacteria can synthesize high concentrations of proline or absorb this compound from the medium where they are growing (Measures, 1975). Another important osmoprotective compound accumulated by bacteria in osmotic stress conditions is glycine betaine (N, N, N-trimethylglycine). Some bacteria are capable of synthesizing it (Galinski and Truper, 1982), however, most of the prokaryotes are incapable of doing so (Moore *et al*., 1987). Another compound that is indicated as a protector for cells submitted to osmotic and matric stress is the disaccharide trehalose (Weisburd, 1988). Csonka (1989) presented a list of bacterial species subject to action of proline or glycine betaine as osmoprotectors. Besides *Pseudomonas*, *Rhizobium* and *Streptomyces* have been reported as potential BCAs of phytonematodes (Hallmann *et al*., 1997; Duponnois *et al*., 1999; Jonathan *et al*., 2000).

Formulation of bioproducts

Wu *et al*. (2011) reported that bacterial populations introduced directly into soils may be negatively affected due to competition from indigenous soil microflora and other stresses. Therefore, for better survival of bacteria while introducing them in the rhizosphere or rhizoplane, an advanced knowledge of the soil environment of that area is essential. Another strategy that can increase the efficacy of bacteria after its application is to use them in alginate capsules. This will guarantee better survival of bacteria in the soil as the granules release bacterial cells slowly and thus efficacy of bacteria against phytonematodes would be increased for a longer period (Bashan, 1986; Trevors *et al*., 1992; Van Elsas *et al*., 1992). Powder formulations can guarantee greater ease in handling and storing rhizobacteria. Kloepper and Schroth (1981) developed a powder formulation containing 20% of xanthan gum and talc to pelletize pieces of seed potato with rhizobacteria. The bacterial population remained viable for 10 months at 4°C at densities over 10^4 UFC/g of formulation. Survival ability of *Pseudomonas* strains treated with saccharose, with or without betaine, and formulated in methylcellulose and talc, was equal to or greater as compared to the cultures suspended in 20% of xanthan gum and talc (Caesar and Burr, 1991). It has also been proved that compounds with high molecular weight, such as saccharose and trehalose, increase the survival of bacteria in dry biopolymers (Costerton, 1985; Mugnier and Jung, 1985).

Bashan and Gonzales (1999) demonstrated that *P. fluorescens* 313 was re-isolated after being maintained in two types of dry inoculant (granules of alginate with and without a supplement of skimmed milk) and stored at ambient temperature for 14 years. The population of bacteria decreased in both types of inoculant, but a significant number of cells survived (10^5–10^6 UFC/g of granule) and the bacteria preserved some of their physiological characteristics. Smit *et al*. (1996) demonstrated encapsulation of *P. fluorescens* in alginate protected against the lytic action of the bacteriophage ϕR2f in the soil. Hall *et al*. (1998) studied the transport and survival of *P. aeruginosa* encapsulated in alginate in the soil. The bacteria showed higher survival rate and greater distribution in the soil.

There are currently various types of formulation prepared from natural materials, such as turf and clay, as well as compounds derived from plants (Roughly, 1970; Thompson, 1980; Walter and Paau, 1993), and the relationship of these with bacterial survival has been much studied (Roughly, 1970; Thompson, 1980). Clays of the montmorillonite type, for example, can protect bacteria in the soil by the creation of microhabitats (Caesar and Burr, 1991).

Formulations act as a tool that a rural producer can use to increase the survival of these microorganisms in soil. These materials can provide a temporary physical protection to bacteria, can create protective microhabitats and supply the bacteria with nutrients (Van Elsas and Heijnen, 1990; Trevors *et al*., 1992; Walter and Paau, 1993).

Cultivation of plants

In four assays carried out in uncultivated soils, the population density of *Pseudomonas putida*

KT2440 fell gradually, remaining below the limits of detection at 200 days after the start of the experiment. Also, when it was introduced in soil planted with *Zea mays* or *Vicia faba*, the bacterium established itself efficiently in the rhizosphere for 12–16 weeks (Molina *et al*., 2000).

Mutants of *Rhizobium* sp. and *P. fluorescens* PsIA12 were inoculated in different botanical species cultivated in the field. Uninoculated plants of maize, pea, lupin and two weeds (*Amaranthus retroflexus*, *Echinochloa crus-galli*) were subsequently grown in this soil. *Rhizobium* sp. re-established itself in the rhizosphere of all the plants after 12 months, whereas *P. fluorescens* strain PsIA12 presented a low capacity for reestablishment in the rhizosphere (Wiehe and Hoflich, 1995). Cao *et al*. (2011) reported that *B. subtilis* SQR 9 showed a good survival in the cucumber rhizosphere; the sites preferred by bacteria were the zone of differentiation, the root hairs and junctions of lateral roots, with 10^6 ufc/g of root in the rhizoplane.

The availability of nutrients for bacteria introduced in the soil is often low (Hattori and Hattori, 1976; Morita, 1986; Van Elsas and Van Overbeek, 1993). Carbon is often unavailable because it is resistant to degradation or at places not always accessible to the bacteria. There are certain plant species that supply compounds that can be used as the lone source of carbon or nitrogen (Goldmann *et al*., 1993). These compounds are nutrient mediators. Therefore, it is also essential to have a prior knowledge of the botanical species that are most favourable to multiplication of nematophagous rhizobacteria to be applied in the soil.

Temperature

Temperature influences metabolic activity, which affects the survival of bacteria (Van Veen *et al*., 1997). According to Garibaldi (1971), for bacteria to reach their maximum population the cells need higher concentrations of iron at high temperatures. *Pseudomonas* strains 7NSK2 and ANPIS presented different optimal temperature bands for growth, survival and production of siderophores. The maximum number of cells of strain 7NSK2 was observed in iron-limiting conditions at 28°C and the maximum production of pyoverdine was at 19°C. Both strains presented good survival at temperatures varying from 4 to 20°C in non-sterile soil for 50 days. The strain ANPIS, however, survived better in temperatures below zero, while 7NSK2 survived better at 28°C (Seong *et al*., 1991). Temperatures above 30°C were not favourable to the activity of *Azotobacter* sp. and *P. fluorescens* (Gupta *et al*., 1995). There are evidences that *Pseudomonas* is capable of surviving in cold climates under roots of winter wheat (De Freitas and Germida, 1992). This is due to the activity of antifreeze proteins present in many bacterial species (Xu *et al*., 1998). Chanway *et al*. (2000) demonstrated that strains of *B. polymyxa* and *P. fluorescens* are promising in terms of their survival in plant roots during harsh winters.

In general, temperatures varying from 20 to 30°C are more favourable to the reproduction and survival of nematophagous bacteria. However, certain bacterial species can adapt to other temperature ranges. Studies should therefore be carried out to pinpoint the bacterial strains that are promising in the biological control of nematodes.

Organic residues

The survival of bacteria can be enhanced if they are protected by some organic matter present in the soil. Stroo *et al*. (1988) reported that non-fluorescent *Pseudomonas* strain B8 can be introduced in plant residues. By this method the microbial community can be exploited to reduce the negative effects of phytopathogens. However, the competitive success of these bacteria depends on environmental conditions. In some instances, nematophagous bacteria that are to be introduced in the soil are not capable of breaking down the organic residues present in the substrate. Under this situation, bacteria are introduced together with another organism capable of using this substrate as a nutritional source and that can be a viable route to ensure the survival and prevalence of the bacteria in question (Stroo *et al*., 1988). B8 strain of *Pseudomonas* was not able to break down the cellulose in the pure culture, which caused a reduction in its population. The situation was reversed when a cellulolytic fungus was added to the medium (Stroo *et al*., 1988).

The attempt by Racke and Sikora (1992) to reduce the influence of biotic and abiotic factors in the survival of *Agrobacterium radiobacter* and *Bacillus sphaericus*, antagonists of *Globodera pallida*, was partially successful. The application of these bacteria in methylcellulose prolonged the time as these bacteria adhered to the roots, and survived even after 28 days in non-sterile dry soil. The addition of simple carbohydrates to cultivated and non-cultivated soils increased not only the survival of *Flavobacterium* sp. P25, but also the indigenous bacterial populations (Mawdsley and Burns, 1994). In an experiment, salicylate (a source of carbon) was added to the soil and broken down by *P. putida*, which led to an increase in the bacterial population and increased its survival (Colbert *et al*., 1993). Hence, it is important to know the most suitable substrate for the activity and survival of bacteria that are to be introduced in the soil. If organic matter is not present, it should be added to increase the efficacy of nematophagous bacteria.

Availability of nutrients

Roesti *et al*. (2006) evaluated the dynamics of the bacterial community during a growing season in three fields of dryland wheat that differed mainly in the management of their fertilization. The principal difference in the agricultural practices was that one field received 60 kg/ha of urea and 20 kg/ha of diammonium phosphate (DAP), as compared to 50 kg/ha of urea and without DAP in the other two fields. The greatest effect on the rhizobacterial community was found at the higher fertilizer level.

Pseudomonas putida strain KT 2440 can use various compounds as sources of carbon and nitrogen to maintain stability in the environment and colonize efficiently in different soils (Ramos *et al*., 1991, 1994; Duque *et al*., 1993). Fluorescent *Pseudomonas* species produce a characteristic fluorescent pigment, pyoverdine, for the acquisition of iron in conditions that are limiting for this element (Ponraj *et al*., 2012). It was observed that a mutant of *P. putida* S11 led to greater survival under stress conditions, i.e. lack of iron, and this greater tolerance was due to the increase in pyoverdine produced by the bacterium. The bacteria also showed better biofilm formation, adhesion to the seeds and competitiveness in colonization of the roots.

A prior knowledge on the availability of nutrients is essential for predicting bacterial activity in the soil (Hattori and Hattori, 1976). The scarcity of nutrients, especially carbon, is one of the main causes of stress for the bacteria in the soil (Hattori and Hattori, 1976; Poindexter, 1981).

Soil humidity

Soil humidity has an influence on the survival of rhizobacteria. According to Stroo *et al*. (1988), there was a decline in the population of non-fluorescent *Pseudomonas* strain B8 after water potential went below −2.5 MPa. It was noted that the bacterium was predominantly on residues when water potential varied from −0.6 to −0.9 MPa. *Pseudomonas* strain B8 was more sensitive to matric stress than to osmotic stress, a feature that has been reported for other gram-negative bacteria (McAneney *et al*., 1982). Simultaneous inoculation of *Pseudomonas* strain B8 and a xerotolerant bacterium in sterile straw resulted in a greater number of bacteria at low water potentials (Stroo *et al*., 1988). Extracellular polysaccharides produced by the competing bacterium probably protected the strain B8 against desiccation, thus increasing its survival (Sutherland, 1972). Likewise, survival rates of *Flavobacterium* strain P25 were increased when the bacteria carried in the inoculants was added to soil with a field capacity between 40 and 50%, compared to wet or dry soil (Mawdsley and Burns, 1994).

There are evidences that some minerals present in the soil can increase bacterial survival in desiccation. In the absence of lactose, minerals with smaller particles (montmorillonite, zeolite and vermiculite) allowed greater survival of two strains of *P. fluorescens* (2-79RN and W4F393) in desiccation, compared to minerals with larger particles (pyrophyllite, talc and kaolinite). Therefore, the addition of lactose to pyrophyllite, talc and kaolinite increased survival of *P. fluorescens* strain 2-79RN (Dandurand *et al*., 1994). Weisburd (1988) reported that enteric bacteria exhibit greater survival during storage under desiccation than *Pseudomonas* species. This happens due to betaine,

a substance with a protective function that is accumulated within bacterial cells under hydric stress, which provide little protection to *Pseudomonas* spp. against desiccation. Other workers are of the opinion that saccharose increases the absorption and accumulation of betaine in the bacteria present in soil (Perroud and LeRudulier, 1985). It is difficult for bacteria that are to be used in the biocontrol of phytonematodes to survive in very wet or dry soils. Therefore, soil must be managed and kept close to field capacity.

Heavy metals

Rau *et al*. (2009) demonstrated tolerance to a number of heavy metals in different rhizobacteria (e.g. *Serratia marcescens* IPSr90 and IPSr82, *Bacillus* sp. IPSr80, *P. aeruginosa* BPSr43, *Arthrobacter ureafaciens* BPSr55) belonging to 18 genera and 38 species. The minimum inhibitory concentration was greatest for arsenic (12.5–20.0 mM) and lead (7.5–10.0 mM). Some rhizobacteria showed tolerance to chrome, zinc, nickel, copper, cobalt and cadmium. The tolerance profiles of rhizobacteria for different metals were placed in the following order, from highest to lowest: arsenic > lead > chrome > zinc > nickel > copper > cobalt > cadmium > mercury. However, these tolerance profiles can vary for other types of bacteria.

Toxic residues

According to Van Veen *et al*. (1997), application of chemical products, which is very common in soils destined for crops, affects the activity of beneficial microorganisms. As new agrochemicals are launched every year, it is necessary to know the effects of these products on bacteria that are to be introduced into the soil.

Inoculum level

The competitive capacity of bacteria can be influenced by the level of inoculum introduced in the soil environment. Lower level of inoculum introduced in the soil leads to a smaller final population of bacteria (Stroo *et al*., 1988). Factors that affect the survival of *Flavobacterium* sp. strain P25 were investigated in laboratory microcosms. Survival rate of strain P25 was greater when the inoculum was applied directly on the plantlet, as compared to the bacterium homogenized in the soil before planting. Survival of bacteria increased with greater inoculum density. After inoculation of 1.1×10^9 individuals/g soil, the number of survivors at 40 days was 12.5 times greater as compared to inoculation at 1.1×10^4 individuals/g soil (Mawdsley and Burns, 1994). Therefore, it is advised to apply a higher level of inoculum in the soil where phytonematodes are to be managed by using antagonistic bacteria.

Collection site and application of rhizobacteria

The selection of a bacterial strain adapted to the soil environment where it is to be introduced represents an important tool for the effective establishment and survival of their population (Van Veen *et al*., 1997; Kumar *et al*., 2007).

11.4 Mechanisms of Resistance

According to Pelczar *et al*. (1997), the spores that are formed within the cell are called endospores and are exclusive to bacteria like *P. penetrans*. The high resistance of bacterial endospores apparently occurs as a result of the dehydration of the protoplasm that occurs during sporulation. Above all, endospores contain considerable quantities of dipicolinic acid (DPA), a compound that is not found in vegetative cells and that can contribute resistance to heat. It was found that DPA is responsible for 5–10% of the dry weight of the endospore and it occurs in combination with calcium. Endospores of some bacteria are so resistant to heat that they can resist boiling for several hours. On the other hand, resistance to chemicals is due to the impermeability of the protoplasmic membrane and layers of the endospore (Setlow, 1994, cited by Chen and Dickson, 1998). Other mechanisms related to long-term survival of endospores are lack of high-energy compounds (ATP and NADH), high content of 3-phosphoglycerate and bivalent

cations (Ca^{2+}, Mg^{2+} and Mn^{2+}), dormancy of enzymes and presence of a thick cortex (Setlow, 1994, cited by Chen and Dickson, 1997).

Chen and Alexander (1973) reported that bacteria with high internal osmotic tension have more capacity to survive in hydric stress conditions, while other vegetative cells may undergo morphological modifications in order to increase their survival ability. According to Roberson and Firestone (1992), the production of exopolysaccharide may also be an important factor in the survival of bacteria submitted to desiccation. Labeda *et al*. (1976) demonstrated that *Arthrobacter globiformis* alters morphologically, taking on a coco-bacillus form and becoming resistant to dried-out soil. It has been observed that gram-negative bacteria introduced in the soil generally tend to reduce their cell size after being submitted to stress conditions (Morita, 1986, 1988). In fact, a reduction in cell size in *P. fluorescens* was observed under conditions of nutritional scarcity (Poindexter, 1981; Van Overbeek *et al*., 1995). Researchers observed no reduction in size of the capsule for *Rhizobium* sp., but there was probably a drop in cytoplasm volume (Bottomley and Dughri, 1989; Postma *et al*., 1989).

Survival of bacteria is also strongly related to their capacity to adhere to plant roots. The glycoprotein complex was responsible for the adherence of a lineage of *P. putida* to the roots of beans and cucumber (Anderson *et al*., 1988; Tari and Anderson, 1988). Generally, the introduction of bacteria in the soil is followed by rapid or, in some cases, gradual population decline. In the case of *Bacillus thuringiensis*, this decline may not affect their efficacy as a BCA of phytopathogens, because proteins with an antimicrobial effect are released into the soil during cell lysis (Beringer *et al*., 1989). However, the efficacy of most bacteria depends on their survival and growth in the soil (Heijnen *et al*., 1993).

11.5 Conclusions

One of the most promising antagonists in the management of phytonematodes is the parasitic bacterium *P. penetrans* and non-parasitic rhizobacteria. Methodologies for sustainable agriculture are proposed with the aim of combining bacterial biocontrol of nematodes with other management methods, so as to act synergistically against nematodes by means of direct suppression, promotion of plant growth and easier colonization of the rhizosphere. To meet the objectives, a prior knowledge of the ecology, biology, survival and action mechanisms of nematophagous bacteria is essential. In this context, it is extremely useful to study the molecular basis related to the mechanisms of bacterial pathogenicity and survival so as to achieve a better and rational phytonematode management.

Another strategy for improving the survival of nematophagous bacteria for the biocontrol of phytonematodes is to study the chemical, physical and biological characteristics of the soil and of the rhizosphere where the nematophagous bacteria are to be introduced. The effects of the soil biotic and abiotic factors on the physiology and ecology of bacteria introduced into the soil are still little known on the micro-scale (pores). Further research in this area needs to be carried out to understand better the physiology of rhizobacteria *in situ*, so that they may be manipulated by means of the advanced molecular techniques. The evaluation of places in the soil with the greatest number of bacterial cells, as well as the local soil conditions that favour growth and survival of these cells, are some other important areas to be studied using molecular tools.

Research into the genetic manipulation of rhizobacteria can improve understanding and management of the rhizosphere ecosystems. Such information can bring new approaches that may be helpful to improve the efficiency of nematophagous bacteria by means of increasing the expression of toxins or enzymes. It may also prove helpful in formulating these bacteria on a commercial level and may ensure good shelf-life and better survival. Safety tests are also required to ensure that genetically modified organisms are not toxic, allergenic or pathogenic to humans and other animals. The antagonists' genetic persistence in the environment and their potential for gene transfer to other organisms needs to be studied. However, some authors are of the opinion that before trying to alter rhizobacteria

genetically for the control of phytonematodes, one should explore the vast range of natural options.

In the case of rhizobacteria, it is important to consider the possibility of applying mixtures of ecologically diverse bacterial species/strains for longer survival and antagonistic activities against nematodes, even under varying ecological conditions. One of the limitations in implementing this practice is soil adversity to bacteria, because such environmental conditions normally act as a buffer to the introduced microorganisms. However, the problem can be overcome by keeping rhizobacteria in a protectant formula. This technology is flexible and adaptable to the physiological needs of many organisms. Future progress in our understanding of nematophagous bacteria, such as their capacity to colonize plant roots, modes of action and interaction with environmental factors, formulations and application as well as survival, can all help in the development of these beneficial microorganisms as secure components in sustainable agriculture.

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12 Nematophagous Bacteria: Field Application and Commercialization

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12.1 Introduction

In our world there is an uncountable variety of nematodes, which are usually classified into feeding types according to their main sources of nutrition. Kimpinski and Sturz (2003) considered them as the most numerous multicellular animals on earth. Among their major groups are plant-parasitic nematodes (PPNs), which can cause considerable losses to a wide variety of economically important crops (see Abd-Elgawad and Askary, Chapter 1, this volume). Chemical control is a widely used option for PPN management. However, chemical nematicides are now being reappraised with a clear aim at the avoidance of their hazards to human beings. It is widely known that many such chemicals demonstrated environmental hazards, high costs, limited availability in numerous countries, or their reduced effectiveness following repeated applications. Presently, only a few chemical nematicides remain, and some of them are likely to be withdrawn in the near future.

Therefore, alternative methods of nematode management have been developed and include one or more components such as using cover crops, green manure, organic or inorganic soil amendments, fallowing, flooding, resistant/tolerant cultivars, hot water treatment, crop rotation and biological control. The use of the latter, biocontrol agents, to replace these chemicals is gaining importance. In this context, nematophagous bacteria have been gaining more consideration as novel, safe and potential tools to provide substantial benefits for phytonematode management. However, consistent performance of these bacteria is influenced by a range of complex intrinsic (microbial), external (host and environmental) and most importantly, integrated factors. These factors make up the specific context in which biocontrol agents are used. Understanding these factors is an essential step towards improving the level and reliability of their biological control activity. If properly handled, these factors can also act synergistically with such agents to increase consistency and efficacy. Therefore rational management decisions can be made only by analysing the interactions naturally occurring among host plant–nematode target–soil–microbial control agent–environment; a five-party interaction (Dong and Zhang, 2006). In fact, the most sustainable approach to nematode control will integrate several tools and strategies. Integrated pest management (IPM) provides a working methodology for pest management

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(e.g. Akhtar, 1997; Kumari and Sivakumar, 2005; Sikora *et al*., 2005; Perry and Maurice, 2013; Usman and Siddiqui, 2013). With better and sound understanding of such complex interactions, microbial control of nematodes will be more fine-tuned through bacterial strain selection and improvement, mass production, formulation, packaging and field application as well. Multidisciplinary collaboration in different facets of nematophagous bacteria for field application and commercialization may include selection of effective bacterial species/strains that can be easily cultured followed by optimum formulation and packaging (for storage, transport and/or field application), successful wide-scale testing and field application (proper equipment, soil application, compatibility with agrochemicals, interaction with other biotic and abiotic factors), efficient quality control and standardization, registration, and sound market assessment (product efficacy, cost, profit margins, shelf-life, ease-of-use, market acceptance, product coverage and stability).

This chapter deals with field application and commercialization of nematophagous bacteria for phytonematode control in terms of the above-mentioned concept. It is the compilation of an updated and comprehensive research effort, which also attempts to discuss and analyse these studies and to stimulate interest in further research and application of these tiny living beings (bacteria): the nematophagous bacteria (Tian *et al*., 2007).

12.2 Formulation and Packaging of Nematophagous Bacteria

Pesticides are chemicals of synthetic, natural or biological (microbial) origin. In general, microbial pesticides such as a bacterium or any antimicrobial agent kill or inhibit the growth of other pests or microbes. The active ingredients are usually formulated as liquids or solids with inert materials, such as the carriers, surface-active ingredients or stabilizers that improve the pesticidal activity. Liquid formulations are usually mixed with water, but in some cases oil. Formulation refers to the preparation of a product from an active ingredient by the addition of certain active (functional) and non-active (inert) substances (Grewal, 2002). Sound formulation of nematophagous bacteria is a vital aspect of their products used to protect plants from phytonematodes and consequently improve plant performance. Formulation is generally intended to improve activity, absorption, delivery, ease-of-use or storage stability of an active ingredient (herein the bacteria and/or their products). Typical examples of pesticide formulation ingredients (additives) include absorbents, adsorbents, anticaking agents, antimicrobial agents, antioxidants, binders, carriers, dispersants, humectants, preservatives, solvents, surfactants, thickeners and UV absorbers (Grewal, 2002). Although the overall concept of bacterial formulations is similar to traditional biopesticide formulations, differences between previously mentioned groups of nematophagous bacteria (see Eissa and Abd-Elgawad, Chapter 9, this volume) may account for their distinct formulations. For example, high oxygen and moisture requirements, vulnerability to temperature extremes and behaviour of entomopathogenic nematode (EPN)-infective juveniles limit our options of choosing the formulation method and ingredients for the symbiotic bacteria within the symbiotic complex. On the contrary, such bionematicides as local strains for local application would eliminate or reduce formulation, packaging, storage and transport costs. Furthermore, only 'fresh' biological agents would be applied, providing improved efficacy of such locally applied bacterial products. This latter offers even the technological promise of producing locally adapted bacterial strains, rather than the 'one size fits all' approach generally used in numerous countries by several companies. Such a technology is currently utilized in Nemaless™ (a commercial suspension of *Serratia marcescens* having 1×10^9 bacterium cells/ml water) to control root-knot (Abd-Elgawad and Mohamed, 2006) and other PPNs (Abd-Elgawad and Aboul-Eid, 2001). The demerit in such a formulation of a locally used product is that the bacteria can be stored up to only a few days in water within tanks. Other factors, such as high transfer-cost and checking human safety of the used *S. marcescens* strain, preclude the deployment of this method worldwide.

The latter factor is gaining much public concern especially with the recently recognized opportunistic human pathogenic bacteria. Another example of biocontrol organisms of questionable safety is the bacterium *Burkholderia cepacia*, a highly successful biocontrol agent of nematode and soil-borne diseases. It was commercialized as Blue Circle™ (Liquid Biological Nematicide). This bacterium is associated with opportunistic lung infections of patients with cystic fibrosis (Handelsman, 2002). Moreover, susceptibility of liquid nematicidal products to microbial contamination and toxicity of antimicrobial agents are factors influencing nematicidal quality during bacterial storage in water. Nevertheless, negating the need to be transported over long distances or stored for a long time, such a liquid formulation may be a suitable method for direct application. Moreover, liquid formulations with either aqueous or mineral oil are user-friendly. These media allow slow, continual growth of the organism or suspend growth to a starved level (Islam *et al*., 2003).

In addition to formulation of nematophagous bacteria in water suspension, these antagonists have been formulated in a variety of ways to control plant pathogens (Burges, 1998). The sporulating, gram-positive bacteria offer a biological solution to the problem of biocontrol agent formulation. Gram-positive bacteria, e.g. the obligate phytonematode-parasitic bacteria *Pasteuria*, offer heat- and desiccation-resistant spores that can be formulated into stable, dry-powder products as well. In fact, *Pasteuria* commercialization has been hindered by two factors intrinsic to their biology: (i) they have a narrow host range although this is not always the case (Mohan *et al*., 2012); and (ii) they are slow growing and difficult to produce *in vitro* (Wilson and Jackson, 2013). For many years, especially before their *in vitro* production, it was only possible to produce *Pasteuria* spp. in living nematodes, which in turn had to be reared in living plants and applied in a related form (Chen and Dickson, 1998). Therefore, *in vivo*-produced *Pasteuria* spp. endospores have been inoculated into pots, microplots and field trials using various sources of inoculum laden with endospores: ground root material, soil, second stage juvenile nematodes (J_2) encumbered with endospores, or endospores in water suspension (Stirling and Wachtel, 1980; Dube and Smart, 1987; Chen *et al*., 1996; Weibelzahl-Fulton *et al*., 1996; Giblin-Davis, 2000; Kariuki and Dickson, 2007). However, formation of *Pasteuria* spp. in some of these tests required careful disturbance of the soil profile to incorporate the endospore for evaluation (Luc *et al*., 2010, 2011a,b).

In 2004, a patent for *in vitro* production of *Pasteuria* spp. was filed by *Pasteuria* Bioscience Alachua (Florida, USA). As a result, the company now produces the product Econem™. This product contains *Pasteuria usgae* (*Candidatus* Pasteuria usgae) and is targeted at sting nematodes (*Belonolaimus* spp.) growing in turf. Granules of product containing *P. usgae* spores and inert material are applied to golf greens using standard granular applicators. It is recommended that at least three sequential applications be made, and each application should be followed immediately by 2.5 mm irrigation. At present, the product is only sold in the USA (Wilson and Jackson, 2013). Luc *et al.* (2010) studied the efficacy of three formulations (nontreated, liquid, or granular) of endospores of *Pasteuria* spp. (produced *in vitro*) on the final population density of *Belonolaimus longicaudatus*. The preparation of liquid formulation was done as a suspension (50 ml) of tap water, growth media and endospores. The granular formulation was prepared by pipetting 1120 µl of growth media and endospores on to 2 g of a clay blank provided by *Pasteuria* Bioscience LLC. Granular and liquid formulations of *in vitro*-produced endospores suppressed nematode population densities by 22% and 59% in the first and 20% and 63% in the second experiment, respectively, relative to the nontreated control. The liquid formulation proved more efficacious than granular formulation. In liquid formulation, the attachment of endospores increased by 147% as compared to granular formulation. Both granular and liquid formulations decreased the population levels of *B. longicaudatus* in the soil, however the liquid treatment was the most efficient (Luc *et al*., 2010).

Another spore-forming bacterium, *Bacillus firmus*, could be commercialized to control phytonematodes in three different products:

(i) talc/kaolin-based formulation at concentration of 10^6 colony-forming units (cfu)/g; (ii) dextrose based/soluble powder (10^9 cfu/g); and (iii) glycerol based/liquid formulation (10^9 cfu/g). Consequently, while the shelf-life of the first product is 6 months, it is extended to 1 year for the latter two products (http://www.agrinaturals.com/NEMOEND-BF.htm).

The multinational company Bayer Crop Science now market two types of *B. firmus* products (Table 12.1), a drench product (Nortica®) and seed treatment products (VOTiVO™ and PONCHO®/VOTiVO™ mix) for use in different markets (Wilson and Jackson, 2013). The drench product is for use on golf greens and efficacy is claimed against all major groups of nematodes that are pests in turf grass (specifically *Meloidogyne* spp., *Belonolaimus* spp. and *Hoplolaimus* spp.). It is recommended that the product be applied 2–7 days prior to planting, and post-application irrigation (minimum of 7.5 cm) is applied. The seed treatment approach allows a much reduced bacterial load to be applied per unit area, in the order of a 1000-fold reduction, as the active ingredient is targeted directly at the rhizosphere of newly developing roots. The *B. firmus* product VOTiVO was originally developed as a standalone product, but now *B. firmus* is predominantly sold as a seed treatment in combination with the insecticide PONCHO (Clothianidin). The PONCHO/VOTiVO mix is also an insecticide and biological seed treatment for use on maize, cotton, sorghum, soybean and sugarbeet for the control of insect pests and PPNs. The PONCHO component makes up 40.3% of the product, compared with 8.1% *B. firmus* and 51.6% other (formulation) ingredients (2×10^9 *B. firmus* cfu/ml and 500 g/l Clothianidin). At present, its products are only sold in the USA. Because, like all *Bacillus* spp. and *Pasteuria*, *B. firmus* produces tough survival-stage endospores, products are claimed to have a 2-year shelf-life when stored under cool dry conditions without refrigeration (Wilson and Jackson, 2013). Other *B. firmus* products such as BioNem, BioSafe and Chancellor are available as wettable powder (see Askary, Chapter 19, this volume).

The non-spore-forming organisms are more difficult to formulate because they do not have much resistance to environmental stresses, unlike the survival mechanisms of spores. The gram-negative microorganisms have a short life and are readily killed by desiccation (Islam *et al*., 2003). They are traditionally formulated into various solid carriers such as wettable powder (WP). SHEATHGUARD™ is a biological fungicide, bionematicide and a plant growth-promoting rhizosphere bacteria (PGPR). Such promising traits resulted from a selected strain of naturally occurring beneficial soil bacteria, *Pseudomonas fluorescens* (IIHR PF-2). The product contains vegetative cells of gram-negative *P. fluorescens*. It is formulated as WP with 1×10^8 cfu/g. SHEATHGUARD is being registered by Indian Pesticides Regulatory Authority – Central Insecticides Board, India. SHEATHGUARD is approved for use in organic agriculture (http://www.agrilife.in/biopesti_microrigin_sheathguard_pf.htm) to control root-knot nematode (RKN), cyst nematode and citrus nematode. This rhizobacterium is commercialized in several formulations (Table 12.2). SHEATHGUARD is stable for a period of 12 months from the date of manufacture.

For the list of commercialized bacterial nematicides in Table 12.1, it should be noted that the presence of a nematicide in this list does not constitute a recommendation. Trade names are used with the understanding that neither endorsement is intended nor is criticism implied of similar products that are not mentioned. Nematicidal application should be used as per instructions on the manufacturer's label. An example of some details for application of *B. firmus* is given in Table 12.3.

Bacillus thuringiensis (Bt) has been used in different formulations as insecticides and occurs as a natural pathogen of PPNs, but less attention was directed to its evaluation as a nematicide (Li *et al*., 2008). The Bt formulations include water dispersible granule, dry flowable, aqueous suspension, granule, technical powder, dust, wettable powder, emulsifiable suspension, aqueous flowable, bait, and oil flowable (Tamez-Guerra *et al*., 1996; US-EPA, 1998; Navon, 2000). The dilution solution used for Bt preparations in bioassays is saline buffer solution (8.5 g NaCl, 6.0 g K_2HPO_4 and 3.0 g KH_2PO_4 per litre, pH 7.0) together

Table 12.1. Some commercial products of bacteria used against phytonematodes.

Product name	Microbial origin	Company or institution	Country	Nematode target	Reference
Econem	*Pasteuria usgae* (or *P. penetrans*)	Bayer CropScience	Multinational	Sting (or root-knot) nematodes	Wilson and Jackson (2013)
Avid 0.15EC (or abamectin)	*Bacillus thuringiensis*	Syngenta Group Company	Multinational	Root-knot and other nematodes	Martin (2013); Martinez and Waltz (2014)
Bionem-WP, BioSafe-WP, and Chancellor-WP	*Bacillus firmus*	Agro Green	Israel	Root-knot and other nematodes including *Heterodera avenae*	Wang and Liu (2004); Askary, Chapter 19, this volume
Nortica VOTiVO PONCHO/VOTiVO	*Bacillus firmus*	Bayer Crop Science	Multinational		Wilson and Jackson (2013)
Deny Blue circle	*Burkholderia cepacia*	Stine Microbial Products	Wisconsin, USA	*Meloidogyne incognita*	Meyer *et al*. (2001); Meyer and Roberts (2002)
BioStart®	*Bacillus chitinosporus*, *B. laterosporus*, *B. licheniformis* (mixture)	Bio-Cat	USA	Root-knot nematodes	Raddy *et al*. (2013)
BioStart™		Rincon-Vitova			
Nemix	*Bacillus subtilis*, *B. licheniformis*	AgriLife/Chr. Hansen	Brazil		Raddy *et al*. (2013)
Nemaless	*Serratia marcescens* produces volatile metabolites toxic to *Meloidogyne* spp. and other PPNs	Agricultural Research Centre	Giza, Egypt	Root-knot and other phytonematodes	Abd-Elgawad and Aboul-Eid (2001); Abd-Elgawad and Mohamed, 2006
SHEATHGUARD (or Sudozone)	*Pseudomonas fluorescens*	Agri Life (Ind Limited (or Agri Land Biotech)	Hyderabad, India	Nematodes such as root-knot, cyst and citrus nematode	http://www.agrilife.in/biopesti_microrigin_sheathguard_pf.htm
Xian Mie	*Bacillus cereus*	XinYi Zhong Kai Agro-Chemical industry Co., Ltd	China	*Meloidogyne* spp. on vegetables	Wei *et al*., 2010; Xiao *et al*., 2013; http://www.zkagrochem.com
Pathway Consortia®	*Bacillus* spp., *Trichoderma* spp., *P. fluorescens*, *Streptomyces* spp.	Pathway Holdings	USA	Phytonematodes	Askary, Chapter 19, this volume
Micronema	*Bacillus* sp., *Pseudomonas* sp., *Rhizobacterium* sp., *Rhizobium* sp.	Agricultural Research Centre	Giza, Egypt	Root-knot and other phytonematodes	Abd-Elgawad *et al*., 2008

Table 12.2. Mass composition and formulations of SHEATHGUARD™, which contains vegetative cells of *Pseudomonas fluorescens* as active ingredient (http://www.agrilife.in/biopesti_microrigin_sheathguard_pf.htm).

Constituent		Function
Pseudomonas fluorescens (W/W)	01.00%	Active
Carboxy methyl cellulose (W/W)	00.50%	Inactive
Moisture (W/W)	08.00% maximum	Inactive
Carrier powder – talc (W/W)	Dispense sufficient quantity	Inactive
Biological composition		
P. fluorescens (cfu/g) powder	1×10^{8}	
Other formulations available		
P. fluorescens (cfu/g) soluble powder	1×10^{9}	
P. fluorescens (cfu/ml) liquid	1×10^{9}	
P. fluorescens lyophilized		

cfu, colony forming units

Table 12.3. Approximate recommended application rates for *Bacillus firmus* seed treatments (adapted from Wilson and Jackson, 2013).

Crop	*B. firmus* (per seed)	*B. firmus* (per ha)	Sowing (plant) rate (per ha)
Maize	2×10^{6}	1.5×10^{11}	74,000 plants
Cotton	2×10^{6}	2×10^{11}	100,000 plants
Sorghum	2×10^{5}	3.7×10^{10}	185,000 grains
Soybean	5.2×10^{8}	1.24×10^{11}	240,000 seeds
Sugarbeet	2.4×10^{6}	2×10^{10}	85,000 seeds

with 0.05% w/v polysorbitan monooleate (Tween 80) as surfactant (Navon, 2000). To test potencies of unknown Bt preparations, an introductory trial with a ten-fold dilution series of the microbial powders and the standard should be conducted. The results of these assays are used to select a narrower dilution in which the LC_{50} will fall approximately midway in the series. Since Bt products were originally used for insect control, mixing their formulation components (spores, crystals, protoxin) with the insect diet renders the microbe available not only to defoliators but also to larvae that penetrate into the diet and feed on inner layers of the medium. In addition, by using this mixing procedure, undesired effects of the adjuvants of the Bt product, mostly fermentation residues, on the larval feeding will be minimized. In contrast, if commercial products are applied to the surface of the diet, the fermentation adjuvants will stack there, possibly causing dose-dependent errors in the test especially at high Bt concentrations (Navon, 2000).

Bt have achieved a good control of important phytonematode genera such as spiral, *Helicotylenchus* spp. (Abd-Elgawad, 1995), citrus, *Tylenchulus semipenetrans* (El-Nagdi *et al*., 2010) and root-knot, *Meloidogyne incognita* (Mohammed *et al*., 2008) using Bt in formulations known as Dipel 2X, Agerin and crude suspension, respectively. Moreover, a bioassay method developed to use the parasporal crystal protein of Bt, YBT-021, against PPNs recorded LC_{50} of 35.62 µg/ml, 75.65 µg/ml, 94.31 µg/ml, 215.21 µg/ml and 128.76 µg/ml for *Meloidogyne hapla*, *Pratylenchus scribneri*, *Tylenchorhynchus* sp., *Ditylenchus destructor* and *Aphelenchoides* sp., respectively (Yu *et al*., 2008). Currently, numerous Bt commercial formulations such as Agerin, Agry, Protecto, Delfin, Dipel 2X and Dipel DF are available (Haggag, 2013) and some of them have proven their nematicidal activity as mentioned above.

Avermectins have nematicidal and insecticidal activities and are in widespread use especially as agents affecting parasitic nematodes (Stretton *et al*., 1987). They are macrocyclic lactones derived from the mycelia of *Streptomyces avermitilis*. Anthelmintics such as ivermectin, selamectin, doramectin and abamectin are derived from the avermectins. Avid 0.15EC is the only abamectin commercial formulation approved for nematode control. While Avid is used for golf greens only, the above-mentioned Nortica® is registered for golf greens, fairways, tees, sod farms, sports fields, cemeteries, industrial grounds, and home lawns (Martin, 2013). However, abamectin is registered as an acaricide and insecticide on ornamentals, but it has been shown to decrease populations of root-knot *Meloidogyne arenaria* (Cayrol *et al*., 1993), foliar *Aphelenchoides fragariae* (Heungens, 1985; LaMondia, 1999) and stem *Ditylenchus dipsaci* (LaMondia, 1999) nematodes. Therefore, other products (Table 12.1), e.g. abamectin/Adagor turf nematicide and miticide (proportion in w/v: Abamectin 2%, 1,2-benzisothiazol-3-one <10%, Sodium hydroxide <10% and other ingredients determined not to be hazardous) launched as insecticides may be used to control PPNs (http://www3.syngenta.com/country/au/SiteCollectionDocuments/Labels/Agador%20SDS%200612.pdf).

Nevertheless, the above-mentioned Bt formulations, designated originally for insect pests, may frequently suffer from a lack of residual activity as an insecticide. This can be due to wash-off by rainfall/wind or degradation by sunlight. Most preparations of Bt on the market are not palatable to insects, which decreases their efficacy (Gillespie *et al*., 1994). Hence, expression of the Bt gene in tobacco and tomato provided the first example of genetically engineered plants for insect control (Barton *et al*., 1987; Vaeck *et al*., 1987). Subsequently, several Bt genes have been expressed in transgenic plants such as tobacco, potato, tomato, cotton, brinjal and rice (Kumar *et al*., 2008). They have been widely adopted in the two field crops currently commercially available, Bt cotton and Bt maize (Shelton, 2012). In parallel, a cysteine proteinase inhibitor expressed in potato plants provided the first demonstration that transgenic resistance to PPNs, e.g. *Globodera pallida* (the potato cyst nematode), can be effective (Urwin *et al*., 2001) as a potentially super substitutional formulation strategy for control of PPNs to express environmentally friendly nematicidal proteins inside the previously known host plant. Then, expression of the non-canonical, non-three-domain Cry protein, Cry6A, in plants could remarkably reduce the brood size of *M. incognita* in plants (Li *et al*., 2007). It is noteworthy that having such multiple proteins to target PPNs is highly sought after since one such protein might be more promising against a specific PPN than another, as is idealistic of this group of insecticides (Hernandez-Martinez *et al*., 2008). Li *et al*. (2008) extended the previous results to a canonical three-domain Cry protein, i.e. Cry5B. Eventually, such genetically engineered crops demonstrating resistance against PPNs might be considered an extraordinary type of bionematicide formulation. It is possible that transgenic plants protected from phytonematode attack could be a common formulation type in the future.

For a potential endophytic bacterium, the successful development of a formulation will generally be based mostly on the understanding of ecology of these bacteria, particularly in the host tissues (Siddiqui and Shaukat, 2003). Basic formulations of endophytic bacteria could be used as seed treatment, root dipping and soil drench with the bacteria *Pantoea agglomerans* MK-29, *Cedecea davisae* MK-30, *Enterobacter* spp. MK-42 and *Pseudomonas putida* MT-19 to significantly reduce early root penetration of *M. incognita* juveniles into tomato roots (Munif *et al*., 2013). To prepare such formulations, the strains tested were first pre-cultured on tryptic soy agar (TSA) for 48 h at 24°C. A loop of the bacteria was then transferred into 100 ml tryptic soy broth (TSB) and incubated on a shaker at 100 rpm and 22–24°C for 48 h. The bacterial suspension was centrifuged at 4°C for 20 min at 4600 × g, and the bacterial cells in the pellet were re-suspended in sterile 1/4-strength Ringer's solution (Merck) and the bacterial suspension was adjusted to optical density $(OD)_{560} = 2.0$ by dilution with Ringer's solution.

The intended symbiotic bacteria are usually found in association with entomopathogenic nematodes (EPNs) as *Steinernema–Xenorhabdus*

and *Heterorhabditis–Photorhabdus* symbiosis (complex). The effect of the symbiotic complex on PPNs has been receiving a great deal of research. It is noteworthy that poor storage and post-application survival are major obstacles to the expanded use of such a complex as bioinsecticides. No nematode formulation meets the 2-year shelf-life requirement of standard chemical pesticides (Grewal, 2002). Although EPN-infective juveniles (IJs) can be stored up to several months in water in refrigerated bubbled tanks, high cost and difficulties of maintaining quality are associated with this method. High oxygen demand, sensitivity of some nematode species to low temperature, susceptibility to microbial contamination, and toxicity of antimicrobial agents are significant factors that can affect nematode quality during storage in water. Therefore, EPNs are usually formulated into non- or semi-liquid substrates soon after they are produced. Formulations for storage and transport may be done with actively moving, reduced mobility or anhydrobiotic nematodes. Formulations with actively moving nematodes include placement of EPNs on or in inert carriers as a convenient means to store and ship small quantities of nematodes. The nematodes in these formulations are fully active and move freely in or on the substrate. The inert carrier formulations may be sponge or vermiculite. Vermiculite formulation is an important improval over sponge. Advantages include a more concentrated EPN product, extended storage stability and more convenient application (Grewal, 2002). Normally, an aqueous EPN suspension is mixed homogeneously with vermiculite, and the mixture is packaged in thin polyethylene bags. Formulations with reduced mobility nematodes may be through physical trapping or metabolic arrest. Formulations with anhydrobiotic nematodes include gels, powders and granules. On the other hand, formulations for application are specifically intended to enhance post-application survival of EPN-IJs in the soil and foliage. Such formulations include desiccated cadavers, capsules and baits. A formulation based on desiccated cadavers coated with clay has been developed that may allow application without cadavers rupturing or adhering together. Macrogels containing encapsulated nematodes have also been suggested as delivery systems for the control of soil and foliar pests. Baits containing infective juveniles, an inert carrier (e.g. corncob grits, groundnut hulls or wheat bran) and a feeding stimulant (e.g. glucose, malt extract, molasses or sucrose) or a sex pheromone have been developed (Georgis, 1990, as in Grewal, 2002). *Steinernema carpocapsae* and *Steinernema scapterisci* have shown particular promise in the baits; because of their 'sit-and-wait' foraging strategy they do not escape the formulation and are more tolerant of desiccation than other species. Grewal (2002) and Shapiro-Ilan and Gaugler (2014) may be consulted for more details about EPN formulations.

Other products of nematophagous bacteria are in the pipeline. For example, Marrone Bio Innovations, Inc. (MBI) (NASDAQ: MBII), announced that it has submitted MBI-302, a biological nematicide, for registration with the United States Environmental Protection Agency (EPA). *Flavobacterium* sp. strain H492 worked favourably against several important PPNs. Strain MBI-302 has two formulations: seed treatment and liquid. It could control *Heterodera glycines* and consequently increase soybean yields (http://www.marronebioinnovations.com/2013/12/marrone-bio-innovations-submits-biological-nematicide-for-epa-registration). The nematicidal potential of *Paenibacillus elgii* strain HOA_{73} against *M. incognita*, under both *in vitro* and *in vivo* conditions, also demonstrated the bacterial ability to control RKN as well as promote plant growth of tomato (Nguyen *et al*., 2013). Furthermore, mixed compost containing increased amounts of chitin and inoculated with *Paenibacillus ehimensis* RS820 was prepared and used as a mixture that could reduce the disease caused by *M. incognita* and improve the growth of tomato plants (Hong *et al*., 2013).

12.3 Factors Affecting Bacterial Survival in the Formulation and Packaging

Interest in using microorganisms to control plant diseases, enhance productivity of agricultural crops, mineral leach, emulsify and

degrade toxic chemicals has led to increased research into factors that affect bacterial survival, activity, dispersal and gene transfer in soil (e.g. Hughes and Poole, 1989; Trevors and van Elsas, 1989; Berg *et al*., 1990; Jain *et al*., 1992). Generally, bacterial survival in the environment is influenced by:

- Abiotic factors such as temperature (bacteria show lower survival rates at high temperatures), relative humidity (RH), relative acidity or alkalinity (high pH values inactivates microbes), moisture content or hydrostatic pressure (high MPa values inactivate bacteria). Among abiotic factors, temperature and moisture content alter the bacterial species in soil, either promoting their persistence or decline. Andreoglou *et al*. (2003) showed that a *Pseudomonas* strain moved faster in the wetter (–0.03 MPa) than in the drier soil (–0.1 MPa), whereas bacterial biocontrol activity effects was more marked at 25 and 21°C than at 17°C);
- Enzymes (proteases, lipase and chitinase; possibly produced by bacteria) killed *Tylenchorhynchus dubius in vitro* and in soil resulted in modifications of the nematode cuticle that resulted in their death (Miller and Sands, 1977);
- Heavy metals (heavy metals have direct effect on the fungal/bacteria ratio with increased metal load) (Rajapaksha *et al*., 2004);
- Nutrients (needed for cell growth and cell proliferation); and
- Biotic factors, e.g. normal microbial fauna and flora, microbial antagonism, type of microbe and host–microbe relationships.

These factors may either have a direct impact on the microbe or cause variation in the physiological state of the cells prior and during growth, affecting cell division/multiplication. This complex gives a research challenge to find the most efficient and stable microbes or strains not only during formulation and packaging but after their release to combat PPNs. Such factors are evidenced by the fact that several beneficial microorganisms have been shown as very effective in the laboratory but only a few have been validated in the field and are receiving attention as products for commercial marketing as bionematicides. Hence, a preliminary assumption for developing a successful bacterial product is the identification of a strain of the microbial agent that will be effective under conditions appropriate to the area in which it will be used. The selected strain must be evaluated in laboratory and field tests and show high target specificity under environmentally favourable conditions. Under such conditions, the bionematicide will perpetuate by releasing more viable infective forms, to sustain further infections in other target organisms. Bacteria that achieve this level of acceptance are attractive to a manufacturer to enable the development of a unique market product. Formulations should contain an easy to use and economical dry or liquid carrier material (e.g. clay pellets, talc, oil emulsifiable suspension etc.) and in some cases contain different formulation components e.g. sucrose, for easy manipulation, high pesticidal activity, biological stability and preventing phytotoxicity.

Comparing the liquid and the dry type of formulations, the former provide a better biological stability with the advantage for foliar spray that the droplets do not evaporate during application. This formulation has good storage stability, high residual activity, is easy to handle and provides effective control of target pests. The market for these liquids remains small as the most important for soil applications to control PPNs is the granular, dust powder and pelletized formulations. Dry formulations are more convenient than liquids for soil applications. For some microorganisms such as the bacterium *Pasteuria penetrans*, wettable granule formulations are preferred for both cost-effective mass production and storage. But this microorganism has major problems for development into a commercial product related to endospore production.

Recently, Pasteuria Bioscience LLC and its predecessor company Entomos, Inc. developed an *in vitro* method of culturing *Pasteuria* spp. that may allow members of this group to be commercialized as biopesticides. According to the producer the *in vitro* harvested endospores of the genus *Pasteuria* were found to be effective or better than *in vivo* produced endospores. In this study endospores of the genus of *Pasteuria*

were produced in bench-top fermenters. A bench-top fermenter or cell-culture bioreactor can be used for a wide variety of organisms, ranging from yeast and bacteria to mammalian, plant and insect cells. The advantage of the method is that compared to the standard use of shake flasks, or roller bottles, in fermentation or in cell culture a bench-top fermenter enables control of pH, dissolved oxygen and other parameters that can cause an inherent limitation in cell yields and protein expression. Research on culture medium optimization and scale-up for bacterial fermentation is an initial challenge before formulation.

The preparation of an appropriate growth medium is fundamental for establishment and multiplication and therefore numerous media may be screened for their capacity to support *in vitro* vegetative forms of *P. penetrans* (Bishop and Ellar, 1991). They used two approaches to produce culture of *P. penetrans*: (i) screening a selection of existing media for bacteria with possible relatedness to *P. penetrans*; and (ii) formulation of a new medium developed from the premise that *P. penetrans* was a fastidious bacterium that might have a requirement beyond the basic heterotrophic need for sources of energy, organic carbon and inorganic salts for some, or all, of the following: the provision of an osmotic support; a pH value equivalent to that of the host; amino acids; vitamins; precursors of nucleic acids and a variety of trace elements. They found that no growth of *P. penetrans* was observed in any of the known media whereas in the new medium ammonium acetate and sucrose were found to maintain bacteria in an apparently healthy stage and enabled survival for up to 1 month. They also noticed that higher concentrations of inorganic salts decreased survival of *P. penetrans*. This work provided the basis for future research on the *in vitro* culture of *P. penetrans* and has helped to determine the conditions necessary for scaling-up fermentation to commercial levels. For any successful process the costs at the industrial scale have to be realistic (Yang *et al*., 2012).

Once efficient production has been achieved issues of preservation and shelf-life need to be addressed. Generally, most bacterial strains can be stored for long periods using cryopreservation at –130°C. *Pasteuria nishizawae* remains stable for 1 year at 4°C (Smith, 2011). Freeze-drying is a popular method to preserve/maintain bacteria and most of the microorganisms are stored in ampoules (Miyamoto-Shinohara *et al*., 2006). Freeze-dried bacteria have been reported to survive for up to 35 years (Rudge, 1991) and the viability of most strains can be maintained for more than 20 years if the cell concentrations are 10^6–10^{10} cell/ml before freeze-drying (Miyamoto-Shinohara *et al*., 2006). For optimization of its production and freeze-drying (Mputu and Thonart, 2013), *P. fluorescens* BTP1 was produced in a bioreactor with difference against-pressure value (0.1 and 0.3 bar for bioreactor 1 and 2, respectively) and cells were harvested during the stationary phase (2 h and 4 h for bioreactor 1 and 2, respectively). Mputu and Thonart (2013) emphasized the role of temperature (4°C better than at room temperature). However, freeze-drying can damage the cells, resulting in loss of viability during processing and subsequent storage (Font de Valdez *et al*., 1983; Palmfeldt *et al*., 2003). Freeze drying can also modify the physical state of membrane lipids and altering proteins (Mputu and Thonart, 2013). Generally, gram-positive bacteria have greater resistance to drying than gram-negative and the difference is due to the structure of the cell surface (Miyamoto-Shinohara *et al*., 2006). Freeze-drying tolerance also depends on: (i) the formulated medium, which should impede freeze-drying damage, enhance storage stability and permit rehydration (Font de Valdez *et al*., 1983); (ii) the physiological state of the cells (different physiological states are created when harvest takes place at different growth phases of cells or under non-optimal growth conditions); (iii) freezing rate (stationary phase cells always survived better than exponential phase ones (Péter and Reichart, 2005)); (iv) freeze-drying and rehydration conditions (Palmfeldt *et al*., 2003); and (v) initial cell concentration as mentioned above (Miyamoto-Shinohara *et al*., 2006).

Temperature-stressed cells have been shown to have greater freeze-drying survival than cells growing at optimal temperature (Broadbent and Lin, 1999). It can be concluded that: (i) freeze-drying medium, carbon starvation and the initial cell concentration contribute to enhanced freeze-drying survival

(Palmfeldt *et al*., 2003); (ii) gram-negative bacteria have poor survival after freeze-drying; and (iii) the super-persistence of a bacterial species after freeze-drying might be due to optimum desiccation (Miyamoto-Shinohara *et al*., 2006).

Climatic conditions may interfere with the populations (growth, survival) of bacterial strains. Under more natural conditions, soil moisture and temperature have major effects on movement of *Pseudomonas* sp. in soil, root colonization and biological activity (effectiveness) as agents for control of soilborne plant pathogens (Vagelas, 2002). Survival in the laboratory, formulation processes and application are critical when considering large-scale production. It is in these areas that there is still much research and development to be done. Nematode-specific parasitic bacteria have been formulated in a variety of ways. The sporulating (endospore-forming), gram-positive bacteria have heat- and desiccation-resistant spores that can be formulated into stable, dry-powder products such as the spore suspensions of *P. penetrans* formulated on dried wettable powder (Nematech Co. Ltd, Japan) (Vagelas *et al*., 2012). The efficacy of *Pasteuria* is influenced by a variety of abiotic factors. A problem with the original preparations of *Pasteuria* 'formulated' in the powder of the roots of a plant used in the *in vivo* method of mass production was the possible effects of plant material on the infection process or the plant growth. Therefore, the bacteria proved more effective when traditional formulation carrier, e.g. root powder containing endospores, was replaced by a carrier that affected the suppression of PPNs. The new technology permits the quick and cost-effective development of multiple strains of *Pasteuria* in traditional commercial fermentation tanks, using easily available growth media *in vitro*, with Econem™ a commercial product and CLARIVA™, a seed treatment nematicide product. Biopesticide manufacturers believe that the technology of both products is opening the door for commercialization of products based on other species of *Pasteuria*.

Besides endospore-forming bacteria other *Bacillus* species are being mass-produced in fermenters. *Bacillus* species produce and secrete quantities of hydrolytic enzymes, extracellular proteases, toxins or Cry proteins. These products act on the target with a specific mode of action such as those producing an extracellular alkaline protease BLG4, which affects nematode cuticle.

Biocontrol of PPNs with rhizobacteria and endophytic bacteria is an acceptable approach but the non-spore-forming *Pseudomonas* species are difficult to formulate as spores are killed by desiccation. Rhizobacteria and endophytic bacteria were traditionally formulated on solid carriers such as dusts, wettable powders and wettable granules or into liquid products. Both *P. fluorescens* (plant growth-promoting rhizobacterium) and *Paecilomyces lilacinus* (egg-parasitic fungi) were tested in talc-based formulations as seed treatment, soil application and in combination against *Meloidogyne graminicola* infecting rice grown under an intensification system (Seenivasan, 2011). *Pseudomonas fluorescens* was so effective as seed/soil application and seed treatment alone against *M. graminicola* that it was similar to the standard chemical carbofuran. Application of *P. fluorescens* as seed/soil treatment resulted in higher grain yield, which was increased 20.6–26.9% over control followed by *P. fluorescens* as seed treatment alone that increased grain yield of rice by 10.7–11.2% over control. However, an economic return per investment was higher when *P. fluorescens* was applied as seed treatment alone (1:8.8–1:12.0 incremental cost benefit ratio) followed by *P. fluorescens* as seed/soil treatment (1:6.2–1:9.7 incremental cost benefit ratio). Talc-based strain mixtures (e.g. *P. fluorescens*) were also effective against *M. incognita* (Jonathan *et al*., 2006; Seenivasan and Poornima, 2010; Kavitha *et al*., 2011) and *Meloidogyne javanica* (Rao *et al*., 2012) under field conditions. Ideally, additives should improve the biocontrol efficacy of the antagonist but should not support the growth of the pathogen or cause any damage to the host plant (Wiyono *et al*., 2008; Sallam *et al*., 2013).

12.4 Field Application

As biocontrol agents, nematophagous bacteria are used to suppress the PPN population density or impact a specific nematode, making it

less abundant or less damaging than it would be otherwise. The general framework of biocontrol against pests includes importation (classical biological control), augmentation (either inoculative or inundative release) and conservation. Classical biocontrol means the intentional introduction of an exotic, usually co-evolved biocontrol agent for permanent establishment and long-term phytonematode control. Augmentation is intended to supplement the naturally occurring population of biocontrol agent by release of natural enemies (http://en.wikipedia.org/wiki/Biological_pest_control). Relatively low numbers may be released at a critical time of the pest size/season (inoculative release) or millions may be applied (inundative release). In inoculative release, we expect that the biocontrol agents will multiply and control the pest for an extended period but not permanently. Usually, we intend to use inundative release to control phytonematodes exclusively by the released organisms. Modification of the soil habitat, environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests is known as conservation biological control.

It goes without saying that many studies on biology and ecology of the nematophagous bacteria over the past 20 years were originally undertaken to discover why these lethal bacteria frequently provided unpredictable field efficacy. The efficacy gap between their promising laboratory results and disappointing field outcomes may be due to their optimum laboratory applications, which are far removed from actual field conditions. Poorer than expected field results may be attributed to one or more of many factors related to application equipment and method, compatibility with agrochemicals and interaction with other biotic and abiotic factors. Here we discuss factors that impact biological control success either directly or indirectly. Since these bacteria are grouped into the abovementioned six major categories, behaviours and biocontrol characters of each group, mentioned in the other related chapters of the book, may help predict the outcome of field releases. Although we have grouped these factors, the distinctions are artificial and constructed for convenience, so when reading about one of such factors, one must remember that they are not independent. For example, abuse of application equipment in a field may render other factors unfruitful even though their being optimum for bacterial efficacy.

12.4.1 Equipment

Nematophagous bacteria can be applied using most conventional liquid application systems designed to deliver pesticides, fertilizers and irrigation. Considerations involved in the selection of an application system are volume, agitation system, pressure and recycling time, environmental conditions and spray distribution pattern (Shetlar, 1999). For example, application equipment for EPN–symbiotic bacterium complex used in different cropping systems and their handling considerations were illustrated by Grewal (2002). Although such a complex was used originally against insect pests, their potentially antagonistic effect on PPN has been reported (see Eissa and Abd-Elgawad, Chapter 9, this volume). Moreover, there is a general similarity in the special procedures and equipment used in the application of biocontrol agents since they are fastidious organisms. Although EPNs with their symbionts can be delivered with low-volume sprayers, pre- and post-application irrigation should be adjusted to compensate for reduced volume. Pre-application irrigation moistens the soil or turf thatch in order to facilitate nematode movement. Post-application irrigation is also necessary to wash any EPNs that may be on plant surfaces into the soil. Studies indicate that 0.25–0.65 cm of post-application irrigation is enough to let the nematodes move into the soil (Shetlar *et al*., 1988). Post-application watering should be conducted before the spray droplets dry; this may lead to treating smaller areas before immediate irrigation in many crop systems.

Contrary to using the, tiny, biocontrol bacteria and/or their filtrate alone, screens and filters need to be large enough to allow passage of the EPN–bacteria complex. The nematode *Steinernema carpocapsae–Xenorhabdus nematophila* complex can pass through sprayer screens with openings as small as 100 µm in diameter, but larger openings are required for

larger EPN species such as *Steinernema glaseri* and *Heterorhabditis megidis* (Grewal, 2002). Therefore, removal of the filters and screens is usual although this will require recalibration of the equipment. High pressure and extensive recycling through the pumping systems can damage nematodes. Most agricultural pumping systems use membrane or roller pumps and do not develop sufficient pressure to damage nematodes or the nematophagous bacteria that are smaller. However, high-pressure hydraulic pumps can develop high internal pressure and may shred the nematodes. In general, nematodes should not be subjected to pressures exceeding 300 psi or 2070 kPa (Grewal, 2002).

The use of application equipment in which bacteria may be subjected to extreme temperatures either in the tank, delivery hose or nozzles should be avoided. For example, *P. penetrans* is a mesophyllic bacterium, with an optimal growth temperature between 28°C and 35°C (Hatz and Dickson, 1992; Serracin *et al*., 1997). Also most irrigation, fertigation and chemigation systems do not empty when they are not in use. Therefore, the nozzles close to the pump source will begin releasing bacteria long before the end nozzle. These systems should be calibrated to determine how much liquid must enter the system before bacteria reach the end nozzle. Such a demonstration was reported for EPN–bacterium complex (Grewal, 2002). When used properly, chemigation (i.e. application of nematophagous bacteria with irrigation water) is an environmentally sound and cost-effective way to control PPNs during the growing season (e.g. Abd-Elgawad and Aboul-Eid, 2001, 2002).

12.4.2 Soil application

Spraying the soil surface is the most common method of application of biocontrol agents. Consequently, edaphic factors such as soil type, moisture and temperature influence bacterial activity as biocontrol agents. Water application immediately following bacterial treatments to secure such optimum factors for their efficacy is usually required as labelled on the bacterial product. Yet, the relative effect of these factors may vary not only between the above-mentioned groups of these bacteria, but also between different species in the same group. Moreover, different formulations even of the same bacterial species may entail variation in both the method of field application and their efficacy as biocontrol agents. A liquid formulation of *in vitro*-produced *P. usgae* (*Candidatus* Pasteuria usgae) endospores gave much better suppression of *B. longicaudatus* population densities than a granular form, but both formulations were superior to the non-treated control (Luc *et al*., 2010).

Seasonal crops, such as tomato, aubergine, squash, etc., are cultivated regularly, which allows for incorporation of nematophagous bacteria such as *Pasteuria* endospores throughout the soil profile and/or plant rhizosphere. Mass multiplication of *P. penetrans* could be obtained in two root-knot nematode-susceptible hosts, tomato and brinjal, with three different doses of root powdered bacterial culture (Gogoi and Neog, 2008). With an increase in inoculum level of root powder culture of *P. penetrans* (1–3 g/m^2), there was corresponding increase in final bacterial spore population/unit weight of root powder. Ceiling final bacterial spore population per unit weight of root powder and utmost decrease of final RKN population level in soil were recorded in the treatment where 3 g/m^2 root powder culture of *P. penetrans* was added in brinjal followed by 3 g/m^2 root powder culture of *P. penetrans* in tomato. Also, in a microplot study, *P. nishizawae* and *H. glycines* were shown to follow the Lotka-Volterra predator-prey model, and numbers of *H. glycines* were reduced below the damage threshold (Atibalentja *et al*., 1998). Noel *et al*. (2010) found that *P. nishizawae* could be successfully transferred to a soybean *P. nishizawae*-free field, became suppressive to *H. glycines*, and was associated with an increase in soybean yield.

Abd-Elgawad and Aboul-Eid (2002) stated that in order to achieve a two-fold goal, i.e. control of both insects and PPNs, necessitates optimal application tactics to maximize field effectiveness of EPNs. This could be through the delivery of the dauer-stage juveniles of EPNs near the plant roots for effective phytonematode control. In the EPN–bacterium complex, EPN activity and survival is favoured by clay rather than sandy-loam soils

(Kaya, 1990). EPNs require water for movement, but only a thin film of water. The effect of soil moisture on nematode host-finding depends upon species with cruise foraging *Heterorhabditis bacteriophora* and *S. glaseri* being more sensitive to moisture extremes than the ambusher *S. carpocapsae* (Grewal, 2002). Maintenance of optimum soil moisture after nematode application enhances activity and efficacy (Shetlar *et al*., 1988). Soil temperature can also affect EPN–bacterium complex efficacy. Warmer temperatures reduce EPN survival but cooler medium reduces their performance (Grewal *et al*., 1994). If soil temperature is above 28°C, a pre-application irrigation is usually recommended to reduce soil temperature before their application (Grewal, 2002).

Each of *S. marcescens* and EPN suspension containing different Egyptian *H. bacteriophora* isolates applied separately through drip irrigation could increase the tomato yield 121.2% and 160.2%, respectively in a sandy loam soil (Abd-Elgawad and Aboul-Eid, 2001). Yield increase in EPN-treated plots was mainly due to insect control. Nevertheless, 1 month after EPN treatment, *M. incognita* population level in soil was undetectable whereas oxamyl or the bacterium treatments showed their lowest soil population levels at season-end. The ring nematode appeared only early in the season but the stunt nematode remained dominant in most treatments throughout the tomato growing season. In another field (Abd-Elgawad and Aboul-Eid, 2002), the influence of four Egyptian heterorhabditid isolates, each applied at 0.55×10^9 infective juveniles/acre, on natural soil populations of PPN infesting watermelon in a newly reclaimed area in Egypt was studied. The isolates EA1, EB5 and EGG reduced *M. incognita* populations significantly ($P \leq 0.05$) at 7 and 21 days after treatments. *Tylenchorhynchus* spp. population densities were not significantly affected in all treatments. Nevertheless, contrary to the untreated check, a reduction in the *Tylenchorhynchus* spp. numbers was a progressive trend 7 and 21 days after treatment by *Heterorhabditis* sp. EA1 and *H. indica* EI3. *Criconemella* spp. were undetected 7 and 21 days after treatment by *H. bacteriophora* EB5, but their population levels were undetectable 7 and 49 days after treatment by *H. indica* EI3 and *H. bacteriophora* EGG, respectively. Significant ($P \leq 0.05$) differences in the spiral nematode population levels occurred only in plots treated by *H. indica* EI3 and *H. bacteriophora* EGG. Differences in the soil application of EPN–bacterium complex as well as their efficacy and economical control of phytonematodes were discussed elsewhere (e.g. Gouge *et al*., 1994; Hu *et al*., 1995; Grewal *et al*., 1997, 1999; Grewal and Georgis, 1998; see Eissa and Abd-Elgawad, Chapter 9, this volume). Due to the presence of overlap between parts of this chapter, we have provided other examples of the soil application throughout the chapter.

12.4.3 Foliar application

Most phytonematode species inhabit the soil and consequently infect plant roots, but a few phytonematodes can cause severe damage to the foliage of a large number of economically important plant species (see Abd-Elgawad and Askary, Chapter 1, this volume). For example, symptoms on flowering ornamentals can vary considerably due to plant and nematode species, but foliage parts may be distorted, stunted and/or killed (LaMondia, 1999). The foliar nematode, *A. fragariae*, can attack more than 250 plant species (Franklin, 1965). The stem and bulb nematode, *D. dipsaci*, can occur in more than 450 hosts (Hooper, 1972). Both species can increase rapidly to tremendous numbers per leaf.

Diagnosis of plants with foliar nematodes can be extremely difficult and is often mixed with other pathogens' infection (LaMondia, 1999). Abamectin can work against pests such as mites, insects and nematodes (Heungens, 1985; Cayrol *et al*., 1993; LaMondia, 1999). Abamectin B1 (Avid 0.15 EC, Novartis, Greensboro, North Carolina) was evaluated against *A. fragariae* infecting *Lamium maculatum* 'White Nancy' (LaMondia, 1999). It significantly decreased *A. fragariae* populations extracted from foliage. Abamectin B1 (Avid 0.15 EC at 0.3 or 0.6 ml/l; 0.005 or 0.011 g a.i./l) could also control *D. dipsaci* infecting *Phlox subulata*. Azalea (*Rhododendron indicum*), begonia (*Begonia* × *tuberhybrida* 'Barbara') and lamium 'White Nancy' naturally infested with *A. fragariae* were sprayed with abamectin,

diazinon, or methiocarb. Results (LaMondia, 1999) revealed that diazinon and abamectin reduced both *A. fragariae* and *D. dipsaci* populations in *Lamium* and *Phlox*; the former compound was more effective. Both compounds may be combined with relevant cultural control tactics to appropriately manage foliar nematodes. Yu *et al*. (2008) developed a bioassay method to use the parasporal crystal protein of Bt against PPNs. Using this method, the parasporal crystal proteins of ten Bt strains showed activity against the nematodes including both *D. destructor* and *Aphelenchoides* sp. Liquid formulation suspensions are generally used in any type of sprayer for foliar application with small portable to large hydraulic sprayers. Foliar applications of pesticides including the microbial pesticides are used to control pests via direct contact or when they feed on the treated plant foliage. *Brevibacillus laterosporus* G4 and *Bacillus nematocida* protease showed nematicidal effects against *Bursaphelenchus xylophilus* (Huang *et al*., 2005; Niu *et al*., 2006; Tian *et al*., 2006). Xia *et al*. (2011) showed that culture by-products of *Bacillus subtilis* strains OKB105 and 69 and *B. amyloliquefaciens* strains FZB42 and B3 were used against *Aphelenchoides besseyi*, *D. destructor*, *B. xylophilus* and *M. javanica*, respectively. The highest mortality rates were observed at 12 h when combinations of *A. besseyi*/B3, *D. destructor*/OKB105, *B. xylophilus*/69 and *M. javanica*/OKB105 induced 10.6%, 27.6%, 35.6% and 100% mortality, respectively.

It seems that *B. amyloliquefaciens* FZB42 might become a commercially successful product for nematode control. Biomex Plus is an example of a commercial product of *B. amyloliquefaciens* FZB42, which also acts as a natural growth stimulant. Biomex Plus is applied as a soil amendment at planting directly to the seed, or incorporated in the soil immediately surrounding the seed, e.g. via an on-planter applicator, or in mixture with liquid placement fertilizers (http://www.omex.co.uk/agriculture/ProductItem.aspx?id=536). The mechanism underlying the effect of *B. amyloliquefaciens* FZB42 against nematodes remains unknown and it is possible that the gene *RBAM_007470* and its related metabolite are involved (Liu *et al*., 2013). In the case of endophytic bacteria, numerous reports have shown that these microorganisms are also capable of inhabiting other environments such as the phyllosphere (Lanna Filho *et al*., 2013). For example *B. amyloliquefaciens* acts efficiently in the control of bacterial speck caused by *Pseudomonas syringae* pv. *tomato* when applied as a foliar spray, but so far studies on the control of PPNs have not been conducted with foliar applications. The same can be concluded for the antagonistic bacteria *B. subtilis* strain QST 713. This strain proved effective in controlling a wide array of fungal and bacterial pathogens when applied as seed treatment or when the bacteria were sprayed on plants. It was formulated as an aqueous suspension (http://www.bioworksinc.com/products/cease-ca.php) but registered only for controlling fungal and bacterial pathogens. Similar results were obtained in earlier studies with the powder formulation of *P. fluorescens* Pf1 (Vidhyasekaran *et al*., 1997). The strain was effective against rice blast pathogen *Pyricularia oryzae* when applied as cell suspensions for seed treatment or foliar sprays. It appears that the bacterium induced systemic resistance (Anita *et al*., 2004). With the above examples we can conclude that more research should be attempted to control PPNs with promising species/isolates of *Bacillus* and *Pseudomonas*.

12.4.4 Compatibility with agrochemicals

Like other biopesticides, nematophagous bacteria are usually directed to locations where other inputs, e.g. chemical pesticides, surfactants (e.g. wetting agents), fertilizers, mineral nutrition and soil amendments, may interact with biocontrol agents. Often it is desirable to tank-mix one or more inputs to save time and money (Grewal, 2002). Endospores formed by bacterial genera *Bacillus*, *Clostridium* and *Pasteuria* are tolerant of exposure to most agrochemicals, so may be utilized with inorganic and organic fertilizers, microelements and several fungicides, herbicides and pesticides. Such endospore-forming bacteria are resistant to heat, desiccation and chemical destruction. Consequently, they have a prolonged shelf-life (more than 1 year) and can be applied to seeds several days prior to seeding (http://www.stimuplant.co.za/content/view/14/28). They can often

be tank-mixed. On the other hand, other groups of nematophagous bacteria such as non-spore-forming organisms do not have much resistance to harsh conditions, e.g. the gram-negative bacteria have a short life and are readily killed by desiccation (Islam *et al*., 2003). Infective juveniles of the EPN–bacterium complex are tolerant of short exposures (2–6 h) to most agrochemicals including herbicides, fungicides, acaricides and insecticides (Rovesti and Deseo, 1990; Ishibashi, 1993) and therefore can often be tank-mixed. However, some pesticides can reduce infectivity and survival of IJs (Zimmerman and Cranshaw, 1990; Patel and Wright, 1996; Grewal *et al*, 1998). Surfactants and other pesticide formulation ingredients may also be toxic to this complex. For example, neem oil is not toxic to IJs at recommended concentrations, but is usually applied with detergents that can be highly toxic (Grewal, 2002). Admittedly, groups of nematophagous bacteria differ in their susceptibility/resistance to agrochemicals and data on even one species of the same group should be used cautiously for other species. For instance, in the case of EPN–bacterium complex, heterorhabditids tend to be more sensitive to physical challenges, including pesticides, than steinernematids (Grewal, 2002). Application of glyphosate herbicide through glyphosate-resistant transgenic plants is popular. Since only some microbial groups can use glyphosate as a source of energy and nutrients while glyphosate is toxic to others, the bacterial equilibrium will switch in plant–endophyte interaction. Kuklinsky-Sobral *et al*. (2005) reported that the cultivable endophytic bacterial community isolated from soybean leaves, stems and roots were *Acinetobacter calcoaceticus*, *Acinetobacter junii*, *Burkholderia gladioli*, *Burkholderia* sp., *Enterobacter sakazaki*, *Klebsiella pneumoniae*, *Pseudomonas oryzihabitans*, *Pseudomonas straminea*, *Ralstonia pickettii* and *Sphingomonas* sp. The DGGE (denaturing gradient gel electrophoresis) analysis from soybean (*Glycine max*) roots, cultivated in soil with and without glyphosate applications (pre-planting), showed few groups that were exclusive for plants cultivated in soil with pre-planting glyphosate addition, e.g. *Herbaspirillum* sp., but others in plants of glyphosate-free soil, such as *Xanthomonas* sp. and *Stenotrophomonas maltophilia*. Two bacterial species (*P. oryzihabitans* and *B. gladioli*) indicated different sensibility profiles to the glyphosate. Therefore, the glyphosate addition may contradict with the equilibrium of such endophytic populations. This latter group comprises various bacterial species with the capacity for plant growth promotion and biocontrol that may be affected. However, this should be evaluated by long-term tests under actual circumstances (Kuklinsky-Sobral *et al*., 2005). Different formulations of the same pesticide also may generally differ in toxicity to these microorganisms. Owing to the continuous introduction of novel active ingredients, carriers and formulations in different market segments and differences in susceptibility and reaction of bacterial species to nematicide formulations, it is difficult to supply comprehensive and up-to-date information.

Some pesticides act synergistically with EPN–bacterium complex and therefore improve EPN efficacy in inundative applications. Imidacloprid (Koppenhoffer and Kaya, 1998), tefluthrin (Nishimatsu and Jackson, 1998) and pathogens such as *Paenibacillus papillae* (Thurston *et al*., 1994) and Bt (Koppenhoffer and Kaya, 1997) act synergistically or additively with the complex (Grewal, 2002). The latter is also compatible with most inorganic fertilizers when introduced inundatively but natural populations are negatively affected. In field studies, organic manure resulted in increased densities of a native population of *Steinernema feltiae*, whereas NPK fertilizer decreased EPN levels regardless of manure applications. Inorganic fertilizers are likely to be compatible with IJs in tank mixes and should not reduce the efficacy of the complex used for short-term control as biological insecticides, but may interfere with attempts to its use as inoculative agents for long-term control (Bednarek and Gaugler, 1997). Generally, organic manure used as fertilizer may enhance establishment and recycling of many microorganisms. Composted manure and urea do not negatively influence *S. carpocapsae* but fresh manure reduces virulence (Shapiro *et al.*, 1997). The above-mentioned compatibility of EPN–bacterium complex with agrochemicals was examined using insect pests as target, but the

existence of such compatibility is possible when PPNs are targeted.

12.4.5 Interaction with other biotic and abiotic factors

It goes without saying that the above-mentioned factors that affect bacterial survival in the formulation and packaging are an integral part of the other biotic and abiotic factors interacting with the bacteria under field conditions.

Interest in developing *Pasteuria* spp. as biocontrol agents has been slow because of problems of specificity and difficulties in the mass production of spore inoculum. Moreover, the biocontrol potential of *P. penetrans* is influenced by other biotic and abiotic factors that have to be understood if it is to be used in an efficient and consistent manner (e.g. Davies *et al.*, 1991; Hatz and Dickson, 1992; Verdejo-Lucas, 1992; Ouri, 1997). Hatz and Dickson (1992) tested *P. penetrans* attachment and development on *M. arenaria* race 1 under different temperatures (10, 20, 25, 30 and 35°C). The best attachment rate of *P. penetrans* endospores occurred on *M. arenaria* (J_2) at 30°C. It developed quicker within the nematode at 30 and 35°C than at 25°C or below. The authors concluded that the body width and length of *M. arenaria* females infected with *P. penetrans* were smaller initially than the same dimensions in uninfected females, but became considerably larger over time at 25, 30 and 35°C. However, probable maximum attachment to J_2 occurred at 25°C, as shown by Freitas (1997). Attachment to J_2s has also been reported to increase with temperature from 15 to 30°C in *M. javanica* (Stirling *et al.*, 1990) and *M. arenaria* (Hatz and Dickson, 1992; Freitas, 1997).

The mass production of *P. penetrans* requires the grasping endospore attachment process on the juveniles (Freitas, 1997). Spore attachment to *M. javanica* was double at 27°C than at 18°C (Stirling *et al.*, 1990). In *P. penetrans*-infested soils, highest attachment to J_2s of *M. arenaria* occurred when soil was maintained at 20–30°C for 4 days (Freitas, 1997). In tests with soil, highest attachment occurred when J_2 were incubated in soil infested with endospores and maintained at 20–30°C for 4 days. Heating J_2 in soil to sub-lethal temperatures (35–40°C) decreased endospore attachment. Incubating *P. penetrans* endospores in soil at 30–70°C for 5 h/day over 10 days resulted in reductions of endospore attachment to nematodes as temperatures of incubation increased to 50°C and higher (Freitas, 1997).

Darban *et al.* (2004) found that temperature affected the development of *P. penetrans* directly. It was observed that relatively high temperature enhanced development of nematodes and, consequently, *P. penetrans.* Likewise, the slower nematode development may indirectly affect the development of *P. penetrans*. Moreover, plants in the glasshouse at fluctuating lower temperatures were healthier than those in the growth room and produced bigger root systems. It seems that healthier and bigger root systems of the glasshouse plants provided more space and less food competition than those in the growth room. Although numbers of juveniles with spores attached were greater when soil temperatures were 25°C and 35°C than at 15°C, and lower in 7–30 than in 0–6 days-old juveniles (Talavera and Mizukubo, 2003). Our studies, undertaken *in vitro*, proved the same idea by which temperature may favour endospore attachment to RKNs (Fig. 12.1).

Talavera and Mizukubo (2003) proved that soil variables, e.g. texture, temperature and moisture, can significantly influence nematode mobility in soil and so affect the possibilities of spore attachment to juveniles as they search for host roots. Moreover, *P. penetrans* occurs more frequently in sand and sandy loam soils than in those with greater amounts of loam and clay (Spaull, 1984; Chen and Dickson, 1998). Mateille *et al.* (1995) showed that the occurrence of *P. penetrans* was greater in sandy soils. They reported that sandy soil may favour endospore attachment to RKNs. However, it was suggested that electrochemical properties of the soil, particularly pH, played a role in attachment to nematode cuticles (Afolabi *et al.*, 1995). Further biochemical investigations suggested that a suitable balance between electrostatic and hydrophobic interactions is necessary to determine whether or not spores attach to the nematode (Afolabi *et al.*, 1995; Davies *et al.*, 1996). Overall, the endospores can persist under harsh conditions (Dickson *et al.*, 1994).

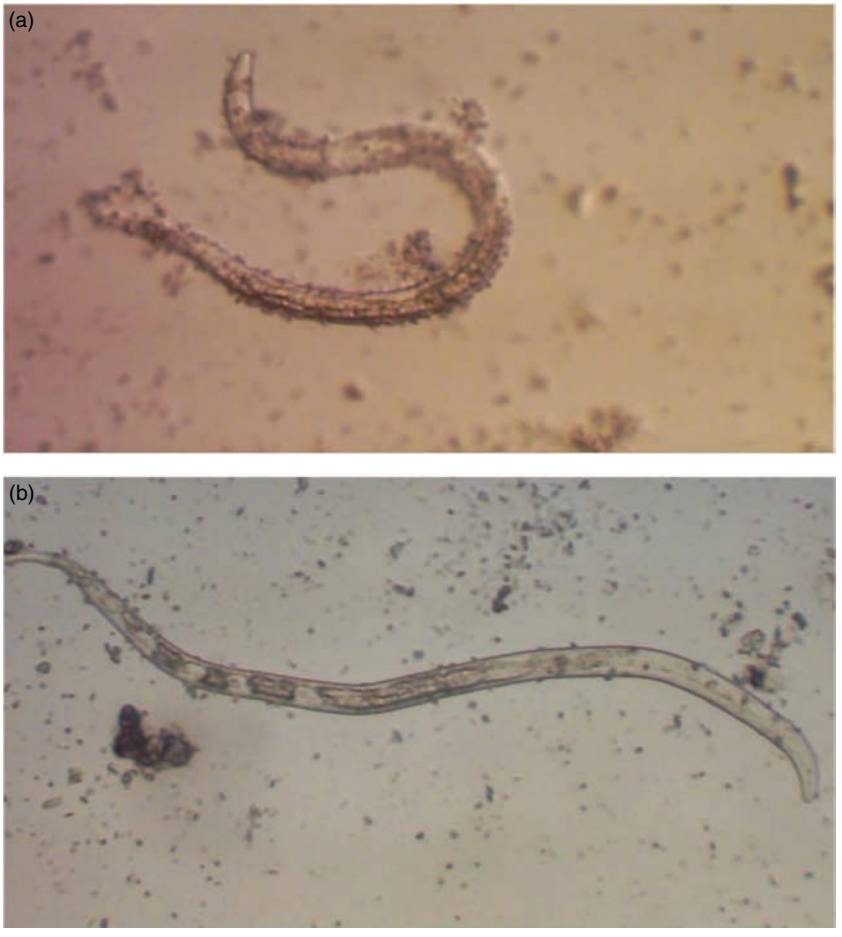

Fig. 12.1. Effect of temperature on the attachment of endospores of *Pasteuria penetrans* to second stage juveniles (J_2) of root-knot nematode, *Meloidogyne* spp. (*in vitro* tests): (a) J_2 encumbered with endospores of *P. penetrans* at 29°C and (b) J_2 encumbered with endospores of *P. penetrans* at 17°C.

Concerning distribution and downward movement of *P. penetrans* in field soil, Cetintas and Dickson (2005) showed how *P. penetrans* endospores move throughout the soil in percolating water. After one addition of water, some endospores were recovered from 25 to 37.5 cm deep. Endospores were detected at the greatest depth, 37.5 to 50 cm, after the third addition of water. The authors concluded that rain or water applications by irrigation are likely to move endospores to deeper levels of the soil, but most endospores remain in the upper 0–30 cm.

Ciancio *et al*. (1994) showed that the host range of the *Pasteuria* group includes 102 nematode genera (although mainly *Meloidogyne* species) and 236 indentified species. Research data have showed that isolates of *P. penetrans* adhere only to a particular species of RKN or to individual populations within a species (Stirling, 1985; Channer and Gowen, 1992; Davies and Danks, 1993); *P. penetrans* was found attached on *Dorylaimus carteri* from Denmark and on *Dorylaimus* sp. from Switzerland (Sayre and Starr, 1988). Endospore attachment to phytonematode species differed among *P. penetrans* isolates (Oostendorp *et al*., 1990) and so did their host ranges (Stirling, 1985). Generally spores adhere randomly on the

nematode body, but Sharma and Davies (1996) showed that spores attach to the anterior or posterior part of nematodes in greater number. Our data suggested that endospores of *P. penetrans* attached particularly to the anterior part of the nematodes in greater numbers (Fig. 12.2).

Sometimes there are large differences in number of spores attached to the cuticle of individuals in a nematode population. This could be due to heterogeneity in the nematode cuticle or in the distribution of the epitope on the spore surface (Brito *et al*., 2003). The appearance of the adhesion epitope first at stage III of sporogenesis and its presence on the parasporal fibres are consistent with an adhesion-related role in the attachment of the mature endospore to the cuticle of the nematode host. Moreover, a certain attribute that makes *P. penetrans* a desirable biological control agent is the synthesis of adhesion-related proteins that was related to endospore maturation (Brito *et al*., 2003). Lectin–carbohydrate interactions have been suggested to be involved in the attachment of *P. penetrans* to its nematode host. These carbohydrate ligands are formed in the process of sporulation (Brito *et al*., 2003) and are believed to perform a primary role in host recognition and attachment (Persidis *et al*., 1991; Davies and Danks, 1993; Davies and Redden, 1997; Charnecki, 1997).

Overall understanding of the processes that lead *P. penetrans* to a successful biological control depend on the effects of certain abiotic and biotic factors such as temperature, moisture, soil, different nematode populations and biochemical aspects occurring during the parasitic development of this promising biological control agent. Likewise, such factors influence other genera/species of nematophagous bacteria but possibly in different ways (see Eissa and Abd-Elgawad, Chapter 9, this volume). For example, three soil bacterial strains, identified as *Chryseobacterium* sp. TFB, could stimulate conidia of the fungus *Arthrobotrys oligospora* to produce a few mycelial traps and conidial traps when cultured with the bacterial cells, but did not produce the traps when cultured with a bacterial cell-free culture filtrate (Li *et al*., 2011).

Fig. 12.2. Endospores of *Pasteuria penetrans* attached to anterior part of the nematodes in greater number.

12.5 Factors Affecting Commercial Success

As with many other biopesticides, commercialization of nematophagous bacteria has experienced highs and lows. Bacteria like *P. penetrans* have been proven to kill and destroy RKNs by their parasitic behaviour while the non-parasitic forms of bacteria can colonize the rhizosphere of the host plant and consequently reduce PPN populations and improve plant growth/yield (e.g. Siddiqui and Mahmood, 1999; Tian *et al*., 2007; Wilson and Jackson, 2013). But for every success, there have been numerous failures. Two basic elements are necessary for nematophagous bacteria to be successful: efficacy and favourable economics. Where nematophagous bacteria have not succeeded, such elements are generally materialized in limited spectrum activity, incompatible performance or unfavourable/slower action by the bacteria. Additionally, developing a biocontrol agent is usually so hard that research has resulted in only a few commercial bacteria for management of PPNs (Meyer and Roberts, 2002; Crow *et al*., 2011). Yet, as the quintessential bionematicide for PPNs, nematophagous bacteria should be poised to fill gaps in management tactics for economically important crops where new 'softer' chemistries are less dominant. Although all such factors can affect the commercial success of nematophagous bacteria as biocontrol agents, the following are those closely related to commercial accommodation of such fastidious organisms. For example, both mass culture and formulation of these bacteria are important for the development of a commercial product. Yet, there has been an

increased tendency to withhold advances in bacterial mass production as 'trade secrets' rather than submitting costly patent applications, a sharp departure from the 'patent-protected technology base espoused by early EPN-bacterium complex production companies' (Friedman, 1990). This is understandable where, unlike formulation technology, patent infringement, related to mass culture of microorganisms, is unapparent in the end-product. Therefore there is a dearth of primary literature, which provides a formidable challenge in documenting and analysing contributions to mass production technology (Gaugler and Han, 2002).

12.5.1 Method of mass-production

Although the development of a commercial product of nematophagous bacteria passes through several stages, the most important factor will be further innovations in production technology that improve cost and availability. It is well known that large-scale production of nematophagous bacteria for use as biopesticides may be through *in vivo* or *in vitro* methods. *In vivo* production (culture in live PPN hosts) usually requires modest technology and low start-up costs. Although the *in vivo* bacterium quality is generally high, cost efficiency is low. This method is ideal for small markets due to the low production volume. Mechanization and streamlining may further improve *in vivo* production. For EPN–bacterium complex, insect cadavers (with EPNs developing inside) are distributed directly to the target site. *In vitro* solid culture of the EPNs on crumbled polyurethane foam offers a mid-position of technology and costs. Their *in vitro* liquid culture is the most sophisticated technology and cost-efficient production method. However, it needs the largest start-up capital. Generally, such methods may be improved through changes in media components, EPN recovery and system design (Shapiro-Ilan and Gaugler, 2014). Abd-Elgawad (2012) adopted a manifold production method to culture *in vitro* EPN–bacterium complex alternately with *in vivo*. The reason behind such a method is to enhance production quantity of EPN–bacterium complex via *in vitro* method and to restore or revive its virulence and infectivity to maximal possible standard through *in vivo* culture.

Before 2004, when a patent for *in vitro* production of *Pasteuria* spp. was filed, *Pasteuria* was produced but only in the body of a nematode as *in vivo* mass production (Stirling and Wachtel, 1980). Pasteuria Bioscience has developed not only a cost-effective method but a revolutionary novel technology which permits the quick development of multiple *Pasteuria* strains in common commercial fermentation tanks, using easily available but promising growth media. This *in vitro* new production technology may enable the launch of a cost-competitive, effective, safe nematicide, which will have the privilege of both replacing environmentally harmful chemicals and significantly growing the nematicide market.

Some researchers (e.g. Friedman, 1990; Ehlers *et al*., 1998) do not consider *in vivo* culture because the prevailing conventional wisdom is that this technology would die out as *in vitro* technologies came online. Yet, there are reasons for the resilience of these *in vivo* methods so far in many countries. For example, since the commercial *Pasteuria* product is only sold in the USA (Wilson and Jackson, 2013), *in vivo* mass-producing *P. penetrans* in other countries relies on producing it in the nematode body on greenhouse-grown plants (Stirling and Wachtel, 1980) and then using the above-mentioned sources of inoculum laden with endospores. Therefore, the plant system should be optimized (Sharma and Stirling, 1991). Serracin *et al*. (1994) reported a hydroponic cultivation system. Suggested improvements for the plant system include using excised or transformed root for culturing (Verdejo and Mankau, 1986; Verdejo and Jaffee, 1988). Chen and Dickson (1998) concluded that its commercial production requires axenic culture. Various media have been tested for their ability to support the growth of isolates of *Pasteuria* spp. (Reise *et al*., 1988). However, Reise *et al*. (1988) did not give the details of the media that they used for cultivation of *Pasteuria* spp., and their study was published only as an abstract (Chen and Dickson, 1998). Williams *et al*. (1989) and Bishop and Ellar (1991) gave detailed descriptions of several microbial media for cultivation of

P. penetrans outside the nematode host. Bishop and Ellar (1991) reported two defined media: one maintained inoculated 'ball-mycelia' of *P. penetrans*, and another gave a small increase in the number of inoculated 'ball mycelia' but lysis resulted. A patent was obtained for a cultivation system that involved adding explanted tissue from *Ascaris suum* to an enriched medium, but this work was never published (Previc and Cox, 1992; in Chen and Dickson, 1998).

This means that there have been many attempts for economic and effective mass culture of the bacteria, which have met ups and downs, making the need to fine-tune a continuous process. Gaugler and Han (2002) also stressed that every facet of *in vivo* technology needs updating to make rearing of EPN–bacterium complex more cost-efficient. Priority needs include demonstration of scalable labour-saving methods, alternatives to gravity settling for concentration and separation of the complex, and an expanded list of inexpensive hosts. Further, mechanisms must be identified for low-volume producers to share in public-funded advances in strain development. This is especially important for EPN–bacterium complex since there are more cottage industry producers than ever before. Despite the reluctance of both industry and academia to invest in *in vivo* methodology, this approach seems unlikely to disappear and, moreover, with assistance could evolve a significant role for local strains in local markets, grower cooperatives, or developing regions.

Nutritional and environmental conditions of the isolated *S. marcescens* 2 and *P. fluorescens* were optimized for mass production to control *M. incognita* on faba bean plants (Kamel *et al*., 2010; Zeinat *et al*., 2010). Such basic investigations and its inherently proprietary nature may stimulate tremendous strides in bacterial mass culture. The challenge now will be to maintain the pace of innovation in a period of stagnant public and private funding. The keys will be: (i) increased academic–industry partnerships; and (ii) a shift in mindset away from developing technologies using the conventional chemical pesticide model (Gaugler and Han, 2002).

12.5.2 Quality control and standardization

Quality control guidelines for products of nematophagous bacteria should be put forward in a standardized manner. The pamphlet should contain guidelines for application procedures and effectiveness. The producer should preferably supervise all measures before shipment (van Lenteren *et al*., 2003). Although quality control tends to be viewed as a final product issue, process control procedures for checking bacterial identity, quality of medium components, stage and quantity of bacterium as active ingredient, and contamination should be established and published in an effort to develop industry-wide standards on quality.

It is noteworthy that market acceptance of biocontrol agent-based products depends heavily on their stability during shipping and storage, as well as ease-of-use and consistent performance under field conditions. Here, stability refers to maintenance of bacterium/its filtrate quality through all stages of the production process. The first step in standardization is aimed at producing reliable active ingredient, i.e. pathogenic bacteria, and/or effective toxin, metabolite, filtrate, etc. against PPNs. Inoculum batches from *in vivo* cultures were produced from stocks of EPN strains and symbiotic bacteria that were stored by cryopreservation (Popiel and Vasquez, 1991) to minimize variation in their pathogenicity among *in vitro* production lots. Subsequent steps were focused on maintaining the viability and pathogenicity of the EPN from fermenter harvesting to end-user application. To ensure this, LT_{50} (lethal time needed to kill 50% of the test insects) or LC_{50} (lethal concentration needed to kill 50% of the test insects) processes were adopted to check the performance standards of IJs, for measuring product stability (Georgis and Kaya, 1998; Georgis, 2002).

Other aspects of product assurance are timing production and optimum storage conditions. For example, EPN–bacterium complex are produced according to seasonal market needs (Georgis, 2002). Most production is accomplished from January to March for products needed in Japan and Europe, whereas those needed for the USA are produced from May to August. For August to

December markets, this complex was produced from March to June. This production spans just 6 months. Optimum storage temperature for formulated bacteria varies according to species; generally, for EPN–bacterium complex, steinernematids tend to store best at 4–8°C whereas heterorhabditids persist better at 10–15°C (Shapiro-Ilan and Gaugler, 2014). Eventually, all stages of the production, handling and application should be adequately developed to serve optimum efficacy of nematophagous bacteria against PPNs and consequently secure commercial success.

12.5.3 Market assessment

Product efficacy, cost, profit margins, competition, shelf-life and ease-of-use are the most important issues that impact the successful introduction and positioning of any bionematicide product into the marketplace.

Product efficacy

All stages involved in the development of the bacterial product should be poised to attain optimum efficacy against PPNs. Basically, there are two approaches to isolate effective bacterial strains: large-scale sampling and location of appropriate environment (Bains, 1993). Large-scale sampling is essentially a random process widely practised in many countries. This 'shotgun' approach has generated thousands of isolates from dozens of countries and every inhabited continent (e.g. Jatala, 1986; Hallmann *et al*., 1997; Bird *et al*., 2003; Guo *et al*., 2008; Aballay *et al*., 2011; Ali, 2012). Because nematophagous bacteria are ubiquitous, even a casual search yields interesting new strains. Unfortunately, there is no evidence that the new strains have desirable traits without laborious and tedious screening in the laboratory and greenhouse. Academic and government researchers usually conduct isolations, so these screens focus on questions of virulence and versatility (Gaugler and Han, 2002). Promising strains are then further tested in field trials. Typically only species and strains that pass these hurdles come to the attention of industry, which assesses suitability for mass production and storage. The alternative approach is a directed search of environments in which the organisms will have had to develop the desired trait. The organisms that break down methane were originally isolated from the soil around a cracked gas main. In nematophagous bacteria, the search is centred on sampling populations of a target pest. This method is still used effectively, e.g. in isolating the *B. xylophilus*-killing bacteria (Morais *et al*., 2011; Proença *et al*., 2011). Likewise, *in vitro* nematicidal activity on *Xiphinema index* of rhizobacteria isolated from grapevine soil was reported (Aballay *et al*., 2011). Similarly, *S. scapterisci–Xenorhabdus* sp. complex was isolated from parasitized mole crickets (Nguyen, 1988). Although this approach is best when performed systematically, most commercial isolates of EPN–bacterium complex were found by chance; that is, they are not used against the original host (Gaugler and Han, 2002). The second component of strain development is strain improvement, which means to modify the genetics of an organism so that it may carry out a process more effectively (Bains, 1993). This approach is heavily emphasized for microbes used in industry, including biological pesticides such as Bt. For example, three Bt mutants were superior to the wild type in controlling *M. incognita* and improving growth of sunflower plants (Eissa *et al*., 2010). Some kinds of biocontrol agents may also be more effective than others. Mukhtar *et al*. (2013) found that both *P. penetrans* and *P. lilacinus* were equally effective against *M. incognita* and caused maximum reductions in number of galls, egg masses, nematode fecundity and build up as compared with *Trichoderma harzianum* and *Pochonia chlamydosporia*.

Many field efficacy trials should be supported through cooperation among various academic researchers and agricultural chemical companies. This will lead to an increase in the awareness of academic researchers, industry, distributors and growers to the use of nematophagous bacteria in phytonematode management strategies. Such trials will distinguish non-valuable from invaluable bacterial species/strains. Through such a cooperation, consistency and high efficacy against PPNs will render commercial success to the product.

Product cost, profit margins, shelf life, ease-of-use, product coverage and stability

The success percentage was substantially reduced from 0.002% in 1995 to 0.0007% in 2008 for chemical control, while costs for development have robustly been increasing (McDougall, 2010). The latter costs for biocontrol represent only a small portion of such expense devoted for chemical control (van Lenteren, 2011). Yet, equal time may be needed to develop a product of each of the two methods but still fundamental economic differences exist. The benefit/cost proportion is more favourable for inoculative biocontrol than for chemical control. For commercial inundative biocontrol it is almost equal, but certainly chemicals are more costly if relevant issues of environmental pollution and health hazards are considered (Pimentel *et al.*, 1980; Pimentel, 2009). Also, issues like pest resistance are low or non-existent in nematophagous bacteria but lower the value of chemical control. Host specificity and the lack of harmful side effects are in favour of biocontrol agents. Nevertheless, the high cost of goods sold continued to be a problem for the failure of some companies like Biosys, where the manufacturer cost of EPN–bacterium complex precluded attaining early goals to penetrate low-value markets such as maize, cotton, potatoes and most vegetables (Georgis, 2002). Even at a low profit margin of 30% for Biosys and the distributor, grower cost was high relative to chemical insecticides. Biosys had successfully produced *S. carpocapsae* and *Steinernema riobrave* in 80,000 fermenters with production exceeding 150×10^3 nematodes/ml. However, contamination, viability of the EPN-infective juveniles and nematode reproduction were recurrent problems that kept the probability of production at 90%. These factors had even greater impact on the production of *S. feltiae*, *S. glaseri* and *H. bacteriophora*, where the probability of achieving successful production did not exceed 85% in 80,000 l fermenters (Georgis, 2002).

Inability to formulate products that are easy to use and have acceptable shelf-life has hindered the development of some biocontrol agents (Georgis and Kaya, 1998; Grewal and Georgis, 1998). On the other hand, other formulations such as calcium alginate for EPN–bacterium complex took 20–30 min to extract the nematodes from the calcium alginate before they could be applied through standard application equipment. Time of extraction (30 min) limited market penetration, however, especially when large-scale treatment was required. Furthermore, the disposal of the framing material (i.e. plastic screen) that held the calcium alginate was burdensome and the number of containers required to treat areas were considered impractical (Georgis, 2002). For such reasons, research should focus on developing more compact product configurations and simpler mixing and application for ease-of-use and consequently further market penetration. Also, we have experienced that some bacterial product coverage is considered inconvenient by many users relative to standard chemical nematicides.

Generally, standard chemical nematicides used in medium- and high-value markets have about 2 years' shelf-life with no requirement for refrigeration. On the other hand, a water-dispersible granular formulation of EPN–bacterium complex has a shelf-life of 5–6 months and requires refrigeration to extend its shelf-life. Such requirements were difficult to accommodate by many distributors and end-users. High relative humidity was needed to maintain the viability of the desiccated nematodes, environments which encourage the growth of fungi and bacteria. Although fungicides were included in the formulation, contamination was a problem in many products. The contamination, in most cases, did not affect nematode viability, but it was a cosmetic problem that was unacceptable to the end-user. Other reasons were related to profit margins (Georgis, 2002).

Market acceptance and penetration

In fact, the relatively limited use of commercial biological control including nematophagous bacteria is due to attitudes of the pesticide industry, farmers, governmental institutions and the biological control community as well as influence of guidelines and regulations as detailed by van Lenteren (2011). The pesticide industry does not show interest in biological control due to the reasons that natural enemies cannot be patented, are host specific, cannot be stored for longer periods,

do not often show compatibility with chemical pesticides and need extra training of sales personnel and farmers. The pesticide industries are not particularly concerned either with sustainable, long-term solutions for pest control and this seems due to the limited patent period on pesticides. In addition, the pesticide industries are always concerned with the development and marketing of new pesticides. This is a matter of concern for biological control, although work of the International Organization for Biological Control (IOBC) has resulted in a European Union (EU) demand of testing side-effects on natural enemies for new pesticides. Such tests, by IOBC and the European Plant Protection Organization (EPPO), are prerequisites for the EU registration of pesticides (EPPO, 2003). In recent years more, but small, companies have started producing natural enemies since numerous chemical pesticides are banned due to environmental issues (Costanza *et al*., 1997; Merino-Pachero, 2007; Pimentel, 2009). If such issues are considered, pesticides should be much more expensive and biological control will be preferable (Pimentel *et al*., 1980; Pimentel, 2009). Many farmers are addicted to using pesticides due to their quick and favoured efficacy. There is a wrong conception among farmers concerning safety of registered pesticides. It is well understood that industries are not interested in such IPM systems where the profit margins are low. Therefore, the governments can impose change by enforcing the use of non-chemical pest control and strongly stimulate use of ecofriendly management options. Guidelines and regulations could and should be drastically simplified and harmonized with such an end in view. Finally, researchers and practitioners of biocontrol should be capable of lobbying and promoting the biocontrol agents for their beneficial traits.

As mentioned above, environmental and health issues confronting the use of chemical insecticides, especially in Europe and North America, may stimulate the use of biological control agents. The development of organic agriculture may also impose more restrictions on chemical control while promoting biological control in greenhouse vegetable production in many regions, e.g. north-west Europe, the USA and China, to name but a few (Merino-Pachero, 2007; Pilkington *et al*., 2010, in van Lenteren, 2011). Food retailers and supermarket chains are increasingly demanding pesticide-free-food and consumers clearly believe that foods produced by using biological control are safer as compared to the use of synthetic pesticides. A recent questionnaire (McNeil *et al*., 2010) indicated people are more content with consuming crops/foods treated by biocontrol agents instead of chemicals to control pests.

Finally, we should exploit the chance of emerging products of nematophagous bacteria in an atmosphere of environmental concern and growing dissatisfaction to conventional chemicals. Therefore, it is wise to carefully examine such products in order not to let them rush to market before developing predictable efficacy and before companies are ready to effectively produce, market or support these products. We should not repeat some failed attempts (e.g. Gaugler, 1997; Georgis, 2002) where prices were much higher than conventional chemicals and/or products had inconsistent formulation and active ingredient content. Key cooperative extension and distributor support should be available to provide sufficient information on proper usage and application and, as a result, end-users will be properly instructed on the characteristics and mode of action of these products. Consequently, more market acceptance and penetration along with improved price performance will be based on advancements in bacterial strain selection, production, formulation and quality control. For example, we should render bacterial products which have more concentrated bacteria and consistent performance as well as longer residual and shelf-life formulations. Additionally, extensive efforts, including seminars, workshops and field days, should be provided to educate growers and distributors on the use of these products for better market penetration.

12.6 Competition

12.6.1 Traditional competition

The traditional competition includes reasons related to the above-mentioned terms such as

ecological reaction or sensitivity of the bacteria to biotic and abiotic factors, formulation, ease-of-use, storage and product cost. Yet, the greatest disappointment is the relative lack of efficacy against PPNs.

Agricultural niche markets are known for their relative lack of competition compared with major markets (Georgis, 2002). It is apparent that microbial nematicides are forced to concentrate their marketing efforts on niche markets because they lack the price performance characteristics necessary to compete in the larger nematicide markets.

On the other hand, chemical nematicides, besides showing rapid effects and being easy to apply, have begun to be withdrawn from the market especially in developed countries due to public health and environmental safety reasons (Schneider *et al*., 2003). Since the search for novel, environmentally friendly alternatives for managing PPNs are gaining importance, nematophagous bacteria may be expanded to major markets in the light of the above-mentioned terms necessary for market penetration and acceptance.

12.6.2 New competition

Reduced toxicity, increased specificity, resistance issues and knowledge-based integrated pest management are key concepts that drove the development of biopesticides (Georgis, 2002). Such a development should be supported by the major entities that can compete in the modern era. Usually, the first step for commercialization of any bacterial pesticide like *B. subtilis* strain QST 713 in any EU country is to be authorized by the EU (http://ec.europa.eu/sanco_pesticides/public/?event=homepage&CFID=1356491&CFTOKEN=89436751&jsessionid=08a0580194ac736cd246779766f497749134TR). Although bacterial biocontrol agents are not widely used against nematodes, the biopesticide market is ruled by the new generation of companies such as Agri Life (An AgriBiotech Enterprise based in Hyderabad, India) and Marrone Bio Innovations, Inc. (Davis, California) and by notable large companies such as Pasteuria Bioscience (Alachua, Florida). This latter produces the product Econem™, which contains *P. usgae* (*Candidatus* Pasteuria usgae) to control sting nematodes (*Belonolaimus* spp.) growing in turf, particularly golf greens. In September 2011, Pasteuria Bioscience and Syngenta entered into a licensing and distribution agreement and, later in 2012, Syngenta acquired Pasteuria Bioscience. Furthermore, the supply of *Pasteuria* products is likely to increase dramatically since, in September 2011, Lonza Group Ltd, which operates a very large-scale fermentation plant, announced a new manufacturing agreement with Pasteuria Bioscience. This agreement secures a process transfer and manufacturing plan to produce *Pasteuria* spores in Lonza's biochemical plant in Kourim, Czech Republic, which has a bioreactor capacity in excess of 500,000 l. Syngenta has announced plans to launch a *Pasteuria*-based seed-treatment product to control soybean cyst nematode in 2014 (Wilson and Jackson, 2013). Admittedly, such companies have made great strides in improving product technology in response to the concerns of conventional nematicides, the regulatory environment and demand for safer products.

Moreover, the information gained from broad advances in molecular biology, nematode ecology, genetics and biochemistry enabled scientists to develop a new generation of control technologies. The new technologies competing with other bionematicides and conventional chemistries can be broadly classified as new chemical products and transgenic plants. These products have already started to have a huge impact on the structure of the agrochemical industry with novel implications for biological nematicides. New chemical products have novel modes of action, very low quantities of active ingredients and narrow specificity compared with conventional insecticides (Georgis, 2002).

12.7 Conclusions and Future Prospects

Growing dissatisfaction with chemical nematicides due to their health and environmental hazards has incited much effort towards economically feasible, agronomically durable and environmentally safe alternatives for such chemicals. Consequently, nematode-antagonistic

microbes and active compounds produced by such organisms are being explored as potential additions to management practices (Meyer, 2003). Many programmes in this area have been investigating nematophagous bacteria as biocontrol agents against PPN. Application or manipulation of nematophagous bacteria is in progress to fill this need. As biocontrol agents, nematophagous bacteria involve the use of beneficial bacteria, their genes and/or products, such as metabolites, that reduce the negative effects of PPNs and promote positive responses by the plant. In this direction, a number of commercial products have been registered both at national and international levels based on different bacterial antagonists. Commercial products such as Nortica, Nemaless, Econem and Sudozone are based on *B. firmus*, *S. marcescens*, *Pasteuria* sp. and *P. fluorescens*, respectively, as active ingredients (Tables 12.1–12.3). There may be some unpublished products sold on the market, but their sell scale is either small, local and/or has not been approved by government while other products are in the pipeline. They can achieve the objective of disease suppression through a number of ways such as antibiosis competition, mycoparasitism, cell wall degradation and induced resistance, plant growth promotion and rhizosphere colonization capability. The most effective biocontrol agent studied to date appears to antagonize the pathogen using multiple mechanisms as in *Pseudomonas*, utilizing both antibiosis and induction of host resistance to suppress the disease-causing microorganisms. As the biocontrol agent represents a living system, a number of difficulties exist to develop such commercial products. These comprise problems with culture and formulation, variable gap between laboratory and field performance, potential negative effects on non-target or beneficial organisms and expectations of broad-spectrum activity and quick efficacy based on practice with synthetic chemical nematicides (e.g. Meyer, 2003; Meyer and Roberts, 2002).

Significant progress has been made during the past two decades in several aspects of bacterial development as bionematicide. This was especially important for the development of *in vitro* mass culture concerning *Pasteuria* spp. and innovative, easy-to-use formulations of numerous bacterial products. Yet, there is a desperate need to poise nematophagous bacteria as effective products against PPN. This trend is currently materialized in a variety of approaches, including studies on their application, improved shelf-life, mass-culture, and interaction with other biotic and abiotic factors as well as integration of biocontrol with other management techniques. As soft power is not political propaganda, it is a mental debate intended to influence public opinion inside and outside the state and can do the work force unable to be done by hard force, beneficial microorganisms such as nematophagous bacteria can induce phytonematode-suppressive soils without chemical nematicides in sustainable agricultural systems.

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13 Novel Bacteria Species in Nematode Biocontrol

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13.1 Introduction

Plant parasitic nematodes (PPN), especially the root-knot nematodes (RKN), are considered a limiting factor in crop production, causing considerable annual losses among a wide variety of crops grown in the world (Sasser and Freckman, 1987). Among the different PPNs, *Meloidogyne* spp. are of much significance. Multiple invasions of plant roots by *Meloidogyne* spp. cause poor development of root system, interfering primarily with water and nutrient absorption. It is clear that *Meloidogyne* spp. can reduce yields substantially, particularly where susceptible crops are grown intensively without fallow periods (Sasser, 1980; Sasser and Freckman, 1987).

Among the different methods used to control PPNs, the main three are: (i) the cultural method (e.g. fallow, crop rotation, sanitation, manuring, water management, trap and resistant crops); (ii) the chemical method (soil fumigants and non-fumigants); and (iii) the biological method. The cultural method, which implies crop rotation with non-host plants, can be an effective method to reduce nematode population densities but non-host crops (e.g. wheat) in most cases are not economical compared to high value crops such as cotton and maize. Chemical nematicides are economical in nematode management but in high value crops only. General public awareness about the environmental hazards, costly chemical nematicides and banning of some soil fumigant chemical nematicides (Lambert and Bekal, 2002) increased the importance of the biological method of control, the most relevant and least damaging approach that is a non-hazardous, ecofriendly, sustainable and cost-effective alternative to chemical nematicides (Shamalie *et al*., 2011). Different microorganisms present in the rhizosphere attack PPNs. The presence of microflora has a profound effect on plants as they regulate the nematode populations in the rhizosphere by influencing both the dynamics of the nematode host and the structure and dynamics of the community of antagonists and parasites in the rhizosphere. Generally, the organisms having a saprophytic phase in their life cycle are most affected by environmental conditions in the rhizosphere, however, obligate parasites are also affected. Nematodes effect the colonization of root by pathogenic and beneficial microorganisms, however, not much information is available of such interactions with the natural enemies of nematodes in the rhizosphere. The quantity and quality of root

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exudates are influenced by nematodes, which in turn affect the physiology of these microorganisms in the rhizosphere; such changes may be used as signals for nematode antagonists and parasites. In order to plan successful biological control strategies, there must be a thorough understanding of these interactions at the population, organism and molecular scale.

One of the most studied and promising examples of natural control of phytonematodes is the bacterial biocontrol agent (BCA). These bacteria occur in soil and cause a high degree of nematode suppression. Researches on soil microorganisms antagonistic to nematodes showed the efficacy of bacterial BCAs such as *Pasteuria penetrans* and *Pseudomonas* spp. (Sayre, 1986; Aalten *et al*., 1998), as a promising, economical and safe strategy in the management of PPNs. This chapter attempts to highlight the use of *P. penetrans* and *Pseudomonas oryzihabitans* in the management of PPN in general and RKN in particular.

13.2 Root-knot Nematodes

13.2.1 A global problem

Root-knot nematodes (*Meloidogyne* spp.) are a major pest of many crops, have wide host ranges and multiply rapidly. *Meloidogyne* spp. are important pests in protected and field-grown vegetable and salad crops worldwide. Farmers are aware of this problem and many resort to the use of pesticides when treating their greenhouses. Pesticide use is an established practice, but international regulations and consumer demands are likely to limit their availability in the future.

Greenhouse production of tomatoes and cucumbers is a major activity in many Mediterranean countries such as Greece, Italy and Spain. Other crops such as melons, beans and vegetables are important field crops. Pest management is a key to yield, quality and profitability in the intensive production systems in greenhouses. These protected growing environments are particularly conducive to pests such as RKNs. Crop rotation is not a viable control option because all the important crops are susceptible to these nematodes, so in the author's view a more sustainable system as a potential biological control agent is needed.

13.2.2 Biology

The second stage juvenile (J_2) of RKN, *Meloidogyne* spp. enter the plant root and establish there with the head in a feeding site of three to eight cells, and gradually swell up in their chosen spot as they progress towards adulthood. During feeding they introduce hormone-like substances into the plant cells, which cause swelling in that area, leading to the formation of galls, or root-knots. Males regain a slender shape and on attaining adulthood they leave the root, but the pear- or melon-shaped adult females remain inside, producing eggs in an egg mass exterior to the roots. The eggs extruded into the soil hatch and the juveniles come out, move into the soil in search of a new host and thus the new cycle begins. Roots infested with RKN usually have visible galls and may exhibit excessive branching. Parasitized plants may be weak and stunted (Southey, 1978, 1986; Agrios, 1997).

13.3 Bacteria

13.3.1 *Pasteuria penetrans*

Pasteuria penetrans (Thorne, 1940) is a naturally occurring parasite of PPNs (Mankau, 1975), showing satisfactory results in a biocontrol strategy of RKNs, *Meloidogyne* spp. (Stirling, 1991). This bacterium is mycelial and endospore forming (Imbriani and Mankau, 1977). The endospores adhere to the cuticle, the outside body wall of the J_2 of *Meliodogyne* (Mankau, 1980). After the nematode (J_2) penetrates a plant root and starts feeding, the bacterium penetrates the nematode cuticle (Mankau and Imbriani, 1975; Imbriani and Mankau, 1977; Sayre and Wergin, 1977) and starts germination, as a result the nematode body is completely filled with spores (Sayre and Wergin, 1977; Stirling, 1991), which in a single nematode is usually on an average of 2.0–2.5 million (Darban *et al*., 2004). These spores are eventually released into the soil.

There are four known species of *Pasteuria* described as endoparasites of nematodes: (i) *P. penetrans* (Thorne, 1940) Sayre and Starr, 1985; (ii) *Pasteuria thornei* (Sayre and Starr, 1988); (iii) *Pasteuria nishizawae* (Sayre *et al*., 1991); and (iv) *Pasteuria usgae* (Giblin-Davis *et al*., 2011). Among these, *P. penetrans* has been extensively studied for its efficiency to control RKN in the field (Bird and Brisbane, 1988; Minton and Sayre, 1989; Davies *et al*., 1991; Dickson *et al*., 1991, 1994; Sturhan *et al*., 2005). However, this bacterial species has two limitations: (i) binding of adhering endospores does not necessarily result in nematode pathogenesis due to narrow host-range specificity (Davies *et al*., 1991, 1994); and (ii) there is no proliferation of bacteria in soil in the absence of nematodes. Numerous studies have been carried out on biological control of RKNs with *P. penetrans*, which include distribution, host range, attachment and host specificity. The researchers have estimated optimal attachment level to be around 5–10 endospores/juvenile, as enough endospores will initiate infection without reducing the nematode's ability for root invasion (Davies *et al*., 1988, 1991; Rao *et al*., 1997). More than 15 endospores (Fig. 13.1) may disable the movement of the nematode, and the

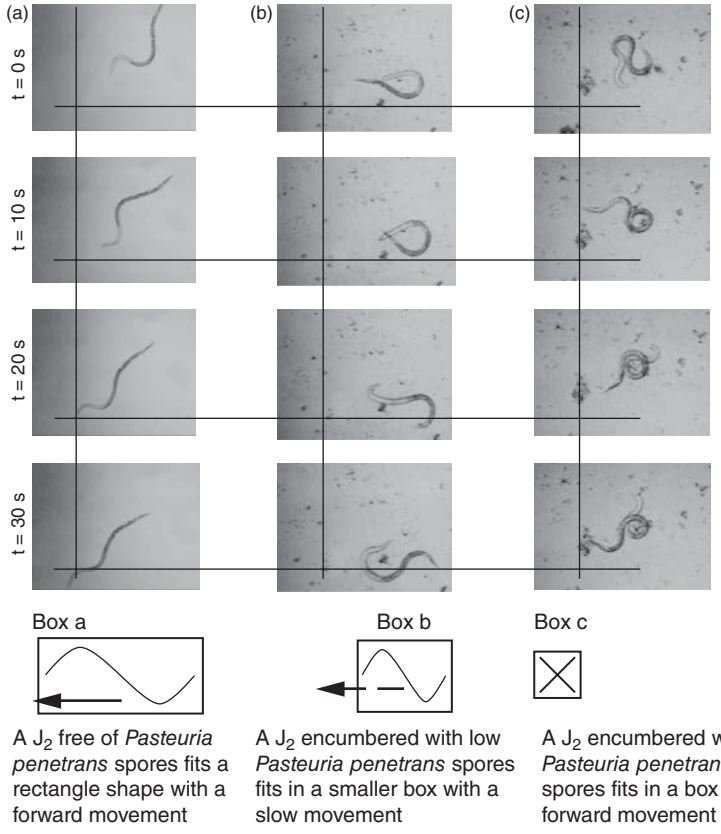

Fig. 13.1. Nematode body wave formation presented as a rectangle shape movement over time. J_2 were encumbered with (b) a low number and (c) a high number of *Pasteuria penetrans* spores or (a) without spores. Arrows indicate the J_2 speed (from Vagelas *et al.*, 2010 and Vagelas, 2011). Data sets (tracking the nematode centroid point and two or four nematode body points) based on the nematode centroid body point were fitted with CurveExpert; the best equation to explain the J_2 body movement without *P. penetrans* spores (a) is the sinusoidal fit, y=a+bcos(cx+d) with correlation coefficients r1=0.9368, r2=0.9396, r3=0.9345 and r4=0.9090 for four random nematodes. When nematodes were encumbered with *P. penetrans* spores there was no sinusoidal movement (b and c).

invasion may be disrupted (Davies *et al*., 1988). That point was not well studied (there are few experimental data) especially on the implication of *P. penetrans* on J_2 movement in soil, an important concept in this context as presented by Vagelas *et al*. (2010) and Vagelas (2011). Moreover, the range and the uniform level of attachment of *P. penetrans* on J_2 are not clear (experimental data show only the bacterium specificity and the J_2 susceptibility to *P. penetrans*).

Recently, the author developed some ideas of a new way to study *P. penetrans*–RKN interactions. In detail, an image analysis system was developed and employed to characterize individual J_2 movement encumbered with or without *P. penetrans* spores (Vagelas *et al*., 2010; Vagelas, 2011). The method used to monitor the movement of free-moving J_2s of RKN (*Meloidogyne* spp.) was based on the video sequence analysis of individual nematodes encumbered with or without the endospores of *P. penetrans*. Software packages were used to grab video images, to process images and to monitor the movement of selected body part positions over time. The useful locations to estimate the locomotion were the nematode centroid point and the rectangular shape area of the nematode. The results indicated that: (i) the normal sinusoidal movement of nematodes is changed when endospores of *P. penetrans* encumber the individuals; and (ii) in all cases nematodes showed a significant greater motility when there was no endospore attachment. Shape parameters of individual J_2 movements were effectively described by the long and short sides of a rectangle. In this model, the longer side of the rectangle was shown as most effective to describe nematode motion. Further, in this research *P. penetrans* 'overdispersion' was modelled using the Poisson and the negative binomial distributions. The negative binomial distribution proved the most appropriate model to fit the observed data sets, considering that *P. penetrans* spores are clumped (Vagelas *et al*., 2010, 2011). The phenomenon of endospores attached in clumps has been neglected as most of the researchers have investigated only for the efficacy of such bacteria and have not followed this up with an investigation for a method of applying the appropriate number of bacteria endospores to a single nematode cuticle to make this microorganism more promising in practice. This probably has many limitations (different type of soil, soil water capacities, soil temperature) in order to be developed into a commercial plant protection product. A similar idea has been previously presented by Evans and Haydock (1993), where pathogens of RKNs have been extensively tested, but it is still unclear whether an obligate parasite such as the bacterium *P. penetrans* might be best in practice.

Future research in biological control is likely to improve our knowledge in bacterium (*P. penetrans*) microbial community function, in particular the structure or densities of bacterial communities in different types of soils or environments that serve as a clump of spores (i.e. 3–5 spores per J_2) and the appropriate biocontrol inoculum required to halt or to reduce the RKN population in soil and in the rhizosphere. The key issue here, apart from the biology, is the mathematics, e.g. probabilities associated with various state changes (Vagelas *et al*., 2012). Mathematical models and algorithms could be tools to solve the problem of the unclear role of the bacterium when applied to fields. Thus, what we really need is to collect all published data for this bacterium and develop with the help of biologists and mathematicians new application strategies for this promising novel bacterium.

Further research on *P. penetrans*–RKN interactions could provide data regarding the movement of nematodes in soil. Figure 13.1 is based on the tracking of the nematode centroid point as mentioned above. The normal sinusoidal movement of nematodes (Fig. 13.1a) is changed when individuals are encumbered with endospores of *P. penetrans* (Figs 13.1b–c). These points have been explained in more detail by Vagelas *et al.* (2010, 2011, 2012). Moreover, the data provide evidence that when the nematode is encumbered with a low number of *P. penetrans* spores, forward movement is impeded (Fig. 13.1b), whereas a heavy attachment of spores blocks nematode motion (Fig. 13.1c). This indicates that there may be little or no root invasion if there are more than 15 spores attached, inferring that spore attachment affects the ability of a J_2 to locate and/or invade a root (Davies *et al*., 1988). How *P. penetrans* changes the motion of a RKN juvenile

on the way into a plant root and how a large number of *P. penetrans* spores adhering to the cuticle of a RKN juvenile probably blocks the juvenile's motion into soil, are illustrated in Fig. 13.1 and in the graph showing the motion analysis and position of nematode body parts over time (Fig. 13.2).

The simple tracking system for extracting data from the four or the two intermediate points of nematode body part showed that nematodes without encumbered spores of *P. penetrans* have a sinusoidal movement producing a double Fourier curve (Eqn 13.1).

$$Y = a + b*\sin(2*pi*(X - e)/w) + c*\sin(4*pi*(x - f)/w) \quad (13.1)$$

This has been represented in Fig. 13.2a, where the Ym1 line presents the oesophagus and the Ym2 line the gut ($R^2 = 86.0$, $P < 0.001$) and in Fig. 13.2b where the tracking points are the head, the oesophagus, the centre of the gut and the tail ($R^2 = 78.4$, $P < 0.001$). Based on the movement of the four body points, the best curve to explain data is also the double Fourier curve. This is a compound of two sine waves as shown in Fig. 13.2, one having half the cyclic period of the other.

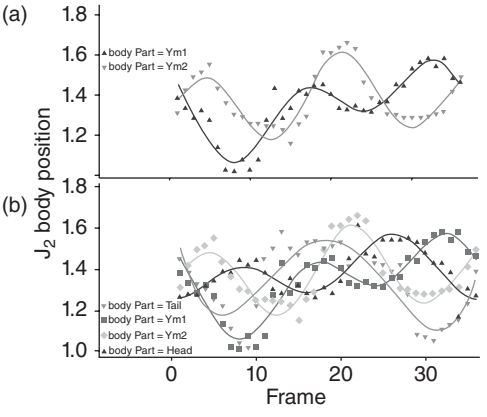

Fig. 13.2. Motion analysis and position of nematode body parts over time. Nematodes were not encumbered with *P. penetrans* endospores. Analysis was performed using GenStat Statistical Package based on (a) Ym1(oesophagus) and Ym2 (gut) data sets and (b) on four nematode tracking points (head, oesophagus, gut and tail). *Y* axis is in proportion to nematode body length, which is equal (in this study) to 1.8 and *X* axis is in frames where 39 frames are equal to 30 s (from Vagelas, 2011).

13.3.2 Other bacteria in biocontrol of nematodes

Besides *Pasteuria*, members of the genera *Pseudomonas* and *Bacillus* show great potential as BCAs of PPNs. They have worldwide distribution and have been reported from at least 51 countries (Siddiqui and Mahmood, 1999; Tian *at al*., 2007). These bacteria act as parasites of several PPNs (Fould *et al*., 2001). These are rhizobacteria (Siddiqui *et al*., 2001; Siddiqui and Shaukat, 2003a), plant-growth-promoting rhizobacteria (PGPR) (Burkett-Cadena *et al*., 2008), endophytic bacteria (Hallmann *et al*., 1995; Siddiqui and Shaukat, 2003b), *cry* genes, gram-positive and spore-forming bacteria (*Bacillus* and related endospore-forming bacteria) (Guo *et al*., 2008) as well as symbiotic bacteria of entomopathogenic nematodes belonging to the genus *Xenorhabdus* and *Photorhabdus* (Shapiro-Ilan *et al*., 2006).

Rhizobacteria

The fluorescent pseudomonads, mainly *Pseudomonas fluorescens*, *Pseudomonas putida* and species of *Bacillus*, are among the most abundant bacteria in the rhizosphere and on the root surface of plants (Pal and McSpadden Gardener, 2006). These bacteria have received considerable attention since the end of the 1970s because they are able to promote the growth of the cultivated plants (Kloepper *et al*., 1980). The mechanisms by which they stimulate the growth of plants are not totally understood but strains such as FZB42 (commercial product RhizoVital®; www.abitep.de/de/download.html?file=tl_files/content/pdf/produktinformationen/Infoblatt_RhizoVital42_fluessig.pdf) have been found to promote growth in tomato and pepper and were able to cause significant reductions in the soil population of juvenile nematodes and the number of galls and egg masses on the root system of tomato (Burkett-Cadena *et al*., 2008). Further to data presented by Gougoulias *et al*. (2010), we believe that microbial activity (Fig. 13.3) has direct effects on plants (provides nutrients), or indirect effects probably related to suppression

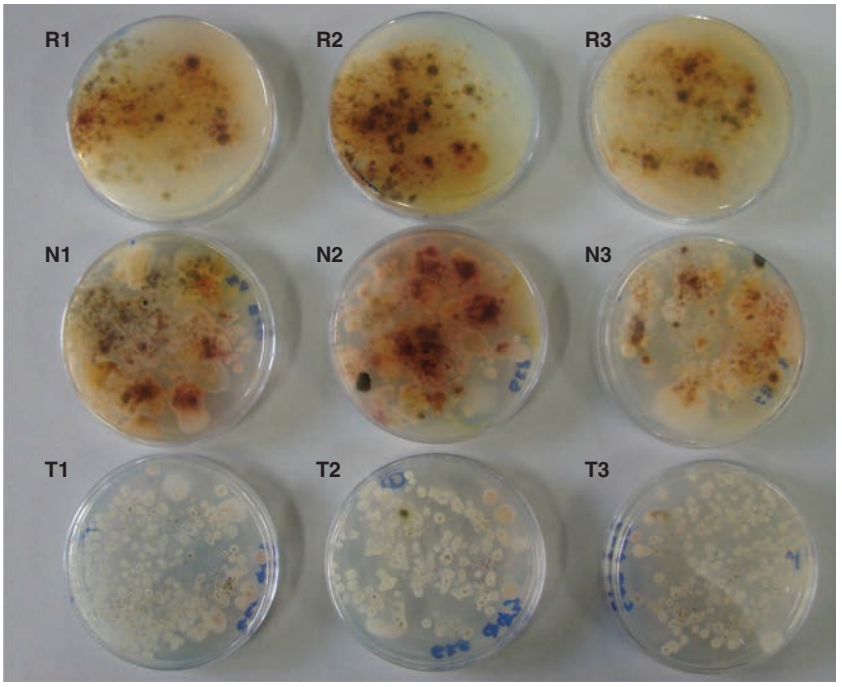

Fig. 13.3. Effect of thiram, oregano or neem on number of bacterial colonies and fungal emergence on agar plates. T1, T2 and T3 are treatments of thiram at application rates 0.1, 0.2 or 0.3 g/50 g soil, respectively; N1, N2 and N3 are treatments of neem at application rates 1.0, 2.0 or 3.0 g/50 g soil, respectively; R1, R2 and R3 are treatments of oregano at application rates 0.2, 0.4 or 0.6 g/50 g soil, respectively.

of soil-borne pathogens (Gravanis *et al*., 2005) or RKNs (Gravanis *et al*., 2006, 2011).

Endophytic bacteria and the majority of rhizobacteria, mainly the strains of *Bacillus* and *Pseudomonas* spp. (Siddiqui and Shaukat, 2003b) as well as bacteria from the genus *Micrococcus* spp., *Arthrobacter* spp., *Curtobacterium* spp. and *Serratia* spp. (Aravind *et al*., 2009) have been identified to induce suppressiveness in PPNs (Hallmann *et al*., 1998; Siddiqui and Shaukat, 2003b; Aravind *et al*., 2009), as well as playing a role in plant growth promotion.

It is also to be noted that despite their different ecological niches, rhizobacteria and endophytic bacteria display some of the same mechanisms for promoting plant growth and controlling phytopathogens, such as competition for an ecological niche or a substrate, production of inhibitory chemicals and induction of systemic resistance (ISR) in host plants (Hallmann, 2001; Compant *et al*., 2005; Pal and McSpadden Gardener, 2006; reviewed in Tian *et al*., 2007).

13.3.3 Bacteria of the genera *Xenorhabdus* and *Photorhabdus*

Bacteria of the genera *Xenorhabdus* and *Photorhabdus* are symbiotically associated with soil-dwelling entomopathogenic nematodes belonging to the genera *Steinernema* and *Heterorhabditis*, respectively (Askary, 2010), and produce several types of secondary metabolites exhibiting antibacterial and/or antifungal activities. There have been previous reports of the isolation and characterization of antimicrobial metabolites including nematophins (indol derivatives), xenorhabdins (dithiolopyrrlones), hydroxystilbenes, water-soluble xenocoumacins (benzopyran-1-one derivatives) and

anthraquinones from cultures of the bacterial genus *Xenorhabdu*s (Maxwell *et al*., 1994).

13.3.4 *Pseudomonas oryzihabitans*

The entomopathogenic nematode *Steinernema abbasi*, isolated from soil in the Sultanate of Oman, has been shown to carry the bacterium *P. oryzihabitans* (Elawad *et al*., 1999) but not in the same manner as members of the genus *Xenorhabdus* (Boemare, 2002). According to Vagelas *et al*., (2007) this bacterium has potential as a BCA not only for insects but also for other target organisms. In particular, *P. oryzihabitans* and its metabolites have been reported as promising biological agents towards various plant root pathogens, such as *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia solani* (Vagelas, 2002; Vagelas *et al*., 2003; Vagelas and Gowen, 2012) and the PPNs *Meloidogyne* spp. (Vagelas *et al*., 2007; Vagelas and Gowen, 2012) and *Globodera rostochiensis* (Samaliev *et al*., 2000; Andreoglou *et al*., 2003). Bacterium activity occurred at 15–28°C and was evaluated for its potential to colonize soil and roots, the production of secondary metabolites and controlling field population of RKNs *in vitro* and *in planta* (Vagelas and Gowen, 2012). *P. oryzihabitans* culture filtrates contain compounds that inhibit hatching of RKNs *in vitro*. Also, *P. oryzihabitans* cells were found decreasing the number of female nematodes and egg masses when applied to soil at the time of nematode inoculation, further demonstrating that the bacteria produce metabolites that have a nematostatic effect upon the infective juveniles.

13.4 Conclusions

Overall in all cases, I believe that any BCA must be a member of the microbial community in the field (crop) in order to achieve profitable disease control. Hence, biological control must involve as a first step the isolation and, later, culturing *in vitro* and application *in vitro* or *in planta* of the specific microorganism. The second step in better understanding of biological control or its failures is the use of the web search machines (Scopus, Web of Science) for tracking similar or other relevant examples from other researchers and finally to conclude and make clear that BCA must be able to contend with a complex physical environment in the field soil or roots or in the leaves. The next step is its application and, finally, the development of a commercial product.

Bacteria in soil and rhizosphere play a significant role in limiting the population growth of PPN but still have an unclear role when applied to fields, and that is why it has proved difficult to develop a bacterial BCA that is effective worldwide against PPNs. In the author's view, PPN suppression can be achieved not only by introducing the specific microorganism or handling and applying them as for chemical pesticides, rather specific knowledge is required to improve the stability and balance in handling of a BCA on the developing PPN population. Perhaps the most encouraging research among different bacteria species has been carried out on the potential of *P. penetrans*. *P. penetrans* compares favourably in the ability of its spores to persist in soil and lacks a need for an organic carrier to ensure successful establishment. Studies on the biological control of RKNs with *P. penetrans* include distribution, host range, recognition, attachment and specificity (Gowen *et al*., 2008). Attachment of from five to ten spores per juvenile is the requirement for a successful parasitism, and has been found sufficient in initiating infection without causing any effect on the root invasion potentiality of the nematode (Davies *et al*., 1988, 1991; Rao *et al*., 1997). More than 15 endospores may cause disability of the nematode in its movements, which ultimately may lead to no invasion (Davies *et al*., 1988). In the author's view this is important, especially in the implementation of *P. penetrans* on J_2 movement in soil. But the most elegant feature is the ability of the bacterium spore to recognize and then infect only the target nematode. Several biotic and abiotic factors influence the antagonistic potential of *P. penetrans* (Davies *et al*., 1991; Hatz and Dickson, 1992; Verdejo-Lucas, 1992; Ouri, 1997). Talavera and Mizukubo (2003) suggested that soil variables, such as texture, moisture and

temperature, were the factors influencing nematode mobility in soil and affecting the chances of spore attachment of *P. penetrans* to RKN juveniles as they search for host roots. Isolates of *P. penetrans* have also been found to adhere only to a particular species of RKN or to individual populations within a species (Stirling, 1985; Channer and Gowen, 1992; Davies and Danks, 1992). These results, i.e. the specificity in *P. penetrans–Meloidogyne* spp. and the heterogeneity of *P. penetrans* field populations are important to enhance the build-up of *P. penetrans* in the field.

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Part IV

Mites

14 Mites as Biocontrol Agents of Phytonematodes

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14.1 Introduction

Reductions in the extent of nematode damage to plants, which may occur without human intervention, are usually attributed to certain biota that decrease nematode numbers in what are termed suppressive soils. These have been reported from all over the world and include some of the best documented cases of natural, effective biological control of nematodes (Kerry, 1997; Sánchez-Moreno and Ferris, 2007). The biological control (BC) of plant nematodes (phytonematodes) has been defined (Sayre and Walter, 1991; Stirling, 1991) as reductions in nematode populations and/or their damage through the activities of organisms other than nematode-resistant host plants. Stirling (2011) later proposed a broader, more ecologically-minded definition, that BC is the action of soil organisms in maintaining nematode population densities at lower average levels than would occur in their absence. Biological control is usually understood to be a scientific as well as a practical approach (and a management tool) in reducing pest numbers and/or their economic, medical and/or veterinary damage, through the activities of other organisms. When it is applied to arthropod pests, BC consists of three strategies, or modes, namely introductions (or classical BC), augmentation and conservation. In the present context, environmental constraints nowadays would almost completely exclude the first strategy, but the possibilities and applications of the other two modes have been discussed below.

Plant-parasitic nematodes are an important component of the soil biological community. Their root-feeding brings them into contact with diverse, numerous soil organisms, and their activities are affected, indirectly and directly, by the soil physical and chemical properties as well as by the prevailing moisture and temperature (Stirling, 2011). The BC of nematodes has been discussed by several authors (Jatala, 1986; Stirling, 1991, 2011; Kerry, 1997) and in the present volume, the controlling agents being mycorrhiza, bacteria and especially fungi. Relatively less has been written about metazoans, such as predatory nematodes, rotifers, tardigrades, Collembola, a few beetles and mites. It is with the latter assemblage that this chapter is concerned.

Mites (Acari) are a vast assemblage of small (usually 0.2–0.7 mm in length) arthropods with four pairs of legs and without conspicuous segmentation (Krantz, 2009); the body appears to be a single unit. The mites have very diverse feeding habits, subsisting on plants

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and their pollen, various animals, whether as predators or parasites, on fungi and on detritus. They are an important component of soils, where species of different lineages feed on nematodes (e.g. Walter, 1988b). Linford and Oliveira (1938) were apparently the first to explore the possible BC of nematodes. Working in Hawaii, they obtained diverse soil biotas that were antagonistic to the root-knot nematode *Meloidogyne marioni* (Cornu). Six (undetermined) mites, all of which were postulated to restrict nematode populations, were among the biota.

14.2 The Nematophagous Acari

14.2.1 Taxonomy

Mites that feed on nematodes have been recognized since 1938 and are referable to several Acarine cohorts, representatives of several major lineages (Krantz and Walter, 2009). These consist of the Mesostigmata, Astigmatina (Astigmata, especially in the family Acaridae), Oribatida (Cryptostigmata), Endeostigmata and Prostigmata. Members of about 30 families contain species that feed on nematodes; lists of taxa were provided by Muraoka and Ishibashi, 1976 (members of 26 families), Walter, 1988a (Endeostigmata), Walter *et al*., 1988 (Mesostigmata), Rockett, 1980 (Oribatida) and by Walter, 1988b (Prostigmata). Additional families such as the the prostigmatic Cunaxidae, mesostigmatid Heatherellidae and the oribatid Phthiracaridae are occasionally being added to this list (Walter and Kaplan, 1991; Walter, 1997; Heidemann *et al*., 2011, respectively). Not all cases discussed in this text refer to phytonematodes, and they are included in order to present a more comprehensive picture of nematophagy by mites.

14.2.2 Modes of feeding

Nematophagous mites differ in their modes of feeding. Members of the Cryptostigmata and some of the Endeostigmata (e.g. Alicorhagiidae), which possess chelate-dentate chelicerae, and some of which actively forage for their prey (Stirling, 1991), ingest the entire nematode, thus being referred to as 'engulfers'. Members of the Mesostigmata and Astigmata possess wounding and sucking mouth parts and they puncture and mangle their prey, ingesting only the body fluids (Fig. 14.1).

However, the mesostigmatid *Hypoaspis calcuttaensis* Bhattacharya is an exception, as it also engulfs the nematode prey (Walia and Mathur, 1995). Some nematophage immatures suck the body fluids of their prey whereas older stages devour the whole body (Muraoka and Ishibashi, 1976). Karg (1983) suggested that specialized nematophagous mesostigmatids can be recognized by their stout cheliceral digits with either a few, large offset teeth or a few large, offset teeth opposed by a saw-like edge of sharp teeth. Walter and Ikonen (1989) showed that predators with such chelicerae also take other prey, and may even feed on fungi. Buryn and Brandl (1992) provided some, albeit weak, support for Karg's hypothesis, noting that more data are needed. A thick, sclerotized cuticle would not protect nematodes against the engulfers, but could hinder fluid-feeding mites (Epsky *et al*., 1988).

14.2.3 Functional groups

Walter *et al*. (1988) placed the nematophagous mites in three functional groups, arranged

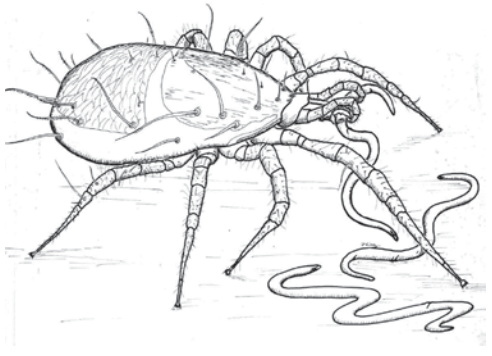

Fig. 14.1. *Macroseius biscutatus* Chant, Denmark and Baker (Mesostigmata: Phytoseiidae) catching a nematode (from the cover of *Phytoseiidae of Florida* by M.H. Muma and H.A. Denmark, Florida Department of Agriculture and Consumer Services, with kind permission from Director E.K. Dyck).

in increasing order of specificity. The general feeders show no preferences for nematodes but can devour any animal they are able to overcome. They consist of members of most of the mesostigmatid families. In the second group are fungivores and detritivores that feed on dead as well as live nematodes (Heidemann *et al*., 2011), and also on algae and fungi; they include the Acaridae and diverse Cryptostigmata as well as many prostigmatids and mesostigmatids. A member of the latter group, the ascid *Gamasellodes vermivorax* Walter, did not initiate oogenesis without the inclusion of nematodes in its diet (Walter, 1987b). For some of these non-specialized predators nematodes are high-quality foods, whereas others often (sometimes preferentially) feed on other diets. Although this may detract from their potential to consume pest nematodes, such a broader diet spectra enables them to survive in the soil for longer periods. Also, notwithstanding their non-specialization, only members of the generalist and fungivorous groups have been suggested as suitable candidates for the control of plant parasitic nematodes. These mites are prevalent in the soil and are very voracious. Some, like the astigmatid *Tyrophagus zachvatkini* Volgin, consume dry, anhydrobiotic nematodes (Walter *et al*., 1986), a diet that confers on the mite the advantage (vis-a-vis biological control) of not being restricted to very humid soil situations. The attraction of another astigmatid, *Sancassania ultima* Samsinak, to undamaged roots, where they hunt for gall-making nematodes (Sell, 1988), is an important attribute of this BC agent.

The third group consists of the specialized predators of nematodes, placed in the families Eviphididae (Mesostigmata), Alicorhagiidae and Alycidae (formerly Bimichaelidae) (both in the Endeostigmata). No member of these specialist mites was accredited with reducing populations of plant parasitic nematodes.

14.3 Prey Finding

Encountering the nematode prey may be fortuitous, due to chance encounters for most feeders, but possibly also directed by various volatiles produced by the nematodes. Walter *et al*. (1986) noted that the astigmatid *Tyrophagus* spp. seemed to be attracted to nematodes by a chemical cue. A sex pheromone was isolated by Jaffe *et al*. (1989) that elicits attractancy and coiling behaviour in the soybean cyst nematode, *Heterodera glycines*. The Ascarosides is a group of highly conserved, small volatile molecules, based on the sugar ascarylose, linked to various fatty acid-like side chains. These volatiles are secreted by many species of soil dwelling, free-living and parasitic nematodes, and mediate various forms of behaviour, and may even effect long-range attraction (Choe *et al*., 2012). Nematode-trapping fungi locate their nematode hosts by 'eavesdropping' on these ascarosides (Hsueh *et al*., 2013). The significance of the signalling ascarosides for specialized nematode predators and/or conditioning them to these compounds could enhance their efficacy. In addition, nematodes wounded by *Tyrophagus putrescentiae* (Schrank) attracted other conspecifics that also fed on the injured prey, probably due to wound-induced volatile compounds (Bilgrami, 1994).

14.3.1 Prey consumption: laboratory studies

Data on the consumption of nematodes were acquired by the three methods appropriate for obtaining trophic information about soil organisms (Walter *et al*., 1991): direct observations, analyses of gut content and experiments and their inferences. Most quantitative data about mite feeding on nematodes (including phytonematodes) were obtained in laboratory studies, usually conducted in Petri dishes with water agar, seldom in pots with soil. Such experiments often show that the mites have a very high rate of predation, seemingly causing close to complete nematode annihilation in the experimental arenas. Consumption differs between species. A *Pergalumna* sp. (Oribatida: Galumnidae) devoured 18.3 second stage juveniles (J_2) of *Meloidogyne javanica* (Treub) per day, and 41.6/day of *Pratylenchus coffeae* (Zimmermann) (Oliveira *et al*., 2007). (For determining the numbers eaten, see below.) Another species of *Pergalumna* devoured up

to three nematodes within a few minutes. The predator grasped its prey at one end, began to chew and pulled the nematode into its oral cavity. Nymphs as well as adults have been found to feed on nematodes (Rockett, 1980). Walter and Ikonen (1989) reported that various mesostigmatids daily consumed 3.8–8.4 individuals of *Acrobeloides* sp. Another mesostigmatid, *Lasioseius subterraneus* Chant, was estimated to be capable of consuming >100 J_2 of the root-knot nematode, *M. incognita* (Kofoid and White) in a day (Walter *et al*. 1993). Imbriani and Mankau (1983) reared *Aphelenchus avenae* Bastian in such dishes and added *Lasioseius scapulatus* (Fox) (which fed only on J_2) for 10 days. The presence of even a single mite in a dish caused a 70% reduction in nematode numbers, and with four mites, prey numbers declined by 99%. *G. vermivorax* consumed nematodes that were twice its body length (Walter, 1987b). At other times only the consumption of egg masses was noted; e.g. Inserra and Davis (1983) reported that another mesostigmatid, which they called *Hypoaspis* nr. *aculeifer* (Canestrini) (now in the genus *Gaeolaelaps*), fed on egg masses of *Meloidogyne chitwoodi*. As to astigmatids, *S. ultima* devoured the egg masses, juveniles and females of *M. javanica*. Sell (1988) named this mite *Caloglyphus* sp. In other experiments Bilgrami (1994, 1997) offered an array of detrivorous, plant-parasitic and predatory nematodes to *T. putrescentiae* and (separately) to *H. calcuttaensis* on water agar in the laboratory. Maximal feeding (after 24 h at 28°C) was on the migratory juveniles of sedentary endoparasitic nematodes. The mites killed 82 and 78% of *Anguina tritici* (Steinbuch) and *M. incognita*, respectively, although some nematode species were little eaten. Minimal feeding (around 40%) occurred on the predatory nematodes, which could have been resistant to predators. Walter *et al.* (1986) reported that adult *Tyrophagus* spp. (0.4–0.5 mm in length) easily fed on large nematodes, up to 0.8 mm long. Walia and Mathur (1995) reported that *T. putrescentiae* voraciously fed on eggs of *M. javanica* within their gelatinous matrix. Moving to prostigmatid mites, *Neocunaxoides andrei* (Baker and Hoffmann) daily consumed about 35 J_2 of *M. javanica* for 5 days in rearing cells (Shoala and El Kady, 2009).

The number of nematodes consumed is affected by the prevailing temperatures. Chen *et al.* (2013) showed that *Blattisocius dolichus* Ma (Mesostigmata: Blattisociidae) devoured significantly more *Radopholus similis* (Cobb) at 25°C than at higher or lower temperatures. A larva of the endeostimatid *Alycus roseus* Koch was observed to feed on 12 nematodes within 30 min (Walter, 1988a).

The prey of various soil mites is sometimes determined by examining the gut boluses of the potential predators and noting any nematode stylets seen therein (Walter, 1988a). This method was further developed by Oliveira *et al.* (2007), who counted the sclerotized buccal stylets and cephalic frameworks of nematodes found in the faecal pellets of *Pergalumna* sp. A qualitative molecular method for detecting nematode remains within the bodies of their predators was developed by Read *et al.* (2006) using specific nematode primers. Heidemann *et al.* (2011), who used this approach, noted that detecting time for various prey may differ between nematodes. They also determined the extent of nematophagy and detriophagy of several species. In the laboratory some nematophagous mites show a functional response to increasing nematode numbers, as the high densities of active prey can stimulate female mites to attack and kill more nematodes than they actually consume (Epsky *et al*., 1988; Bilgrami, 1997).

As observed by scientists, many nematophagous mites have been found to accept other prey too, but prefer nematodes. A species of *Gamasiphis* (Mesostigmata: Ologamasidae) developed faster when feeding on the citrus parasitic nematode, *Tylenchulus semipenetrans* Cobb, than on an acarid mite, and its fecundity increased on the nematodes while decreased on the acarid (El-Banhawy *et al*., 1999). Some omnivorous mites develop more rapidly, lay more eggs and may develop faster when nematodes are included in their diet (Walter, 1988b). The cryptostigmatid *Pilogalumna* sp. produced 8–16 eggs when given fungi and algae, but when nematodes were added to this diet, 20–26 eggs were produced (Walter, 1987a). In other cases nematodes provide only suboptimal food. *Lasioseius athiasae* Nawar and Nasr produced on an average

0.35 eggs/day when feeding on *M. incognita*, as compared to 3.21, 1.63 and 1.26 eggs/day on mite or insect egg prey (Abou-Awad *et al*., 2001). In contrast, *L. subterraneus* voraciously devours nematodes (Walter *et al*., 1993). This shows that the extent of feeding on and utilization of nematodes may differ amongst members of acarine genera of different lineages. It has been found that the extent of nematophagy differs between species of *Pergalumna* (Rockett, 1980) and *Tyrophagus* (Walter *et al*., 1986). Only 11% of *T. putrescentiae* has been noted to feed on nematodes in the presence of other diets (yeasts and algae) as compared to *T. zachvatkini* (23%) and *Tyrophagus similis* Volgin (56%) (Walter *et al*., 1986).

14.4 Nematophagous Mites in Pots and in the Soil

As noted, the number of nematodes killed by the various mites in the laboratory differs greatly, being affected by predator as well as prey characteristics. In the field these environmental conditions, especially the ambient humidity, play a major role, as most soil-inhabiting acarine predators perform best in high relative humidity (but not in water-clogged soils) and medium temperature. Nematode location in the various soil strata affects their susceptibility to the predators. Those nearer to the surface are more likely to be found by the mites, whereas those in deeper layers are relatively protected. Other factors include the prey stages taken (eggs, juveniles and/or adults), and the predators' different feeding modes as described above. The mites' hunting modes, whether foraging or being more sessile, affect their prey finding. Shoala and El Kady (2009) inoculated 500 J_2s of *M. javanica* into pots with tomato seedlings and added 20, 40 and 60 *N. andrei* females. After 5 days the mites had significantly reduced the number of nematode galls on the tomato roots (Fig. 14.2).

Batches of 500 *B. dolichus* reduced a population of 1000 *R. similis* per pot containing *Anthurium* seedlings. Nematode reduction was by 66% within 10 days (Chen *et al*., 2013). In greenhouse conditions, Sharma (1971) explored the effect of three Mesostigmata on the population of nematodes in pots, each inoculated with 25 adults of *Tylenchorhynchus dubius* (Butschli). The predators were *Rhodacarus roseus* Oudemans, *Pergamasus truncatellus* (Berlese) and *Gaeolaelaps aculeifer*. After 4 weeks the former two predators had little effect on final nematode numbers, whereas *G. aculeifer* reduced nematode populations by almost as much as all three predators together. This mite had another subtle but important influence on the nematode populations, as it appeared to prefer pest juveniles. Another mesostigmatid, the laelapid *Cosmolaelaps simplex* Berlese, reduced the numbers of *T. semipenetrans* by 62% (from an average of 4011 to 1525 J_2/100 cm^3 soil) in pots bearing citrus seedlings, after 90 days (Al Rehiayani and Fouly, 2005).

The effect of soil type on the predation of *T. putrescentiae* was examined by Walia and Mathur (1997). They placed 5000 J_2 of *M. javanica* into 150 ml flasks containing different soil types, i.e. sand, loam and clay, and added five adult mites to each flask. Most predation (determined as least prey survival) occurred in the sandy soil, less in the loam and least in the clay (Fig. 14.3).

Schneider *et al*. (2004) developed a qualitative method for determining the diets of soil organisms by measuring the amount of the stable isotope ratios of $^{15}N/^{14}N$ in their bodies. Predatory mites, such as the nematophagous *Pergalumna* spp., had higher ^{15}N

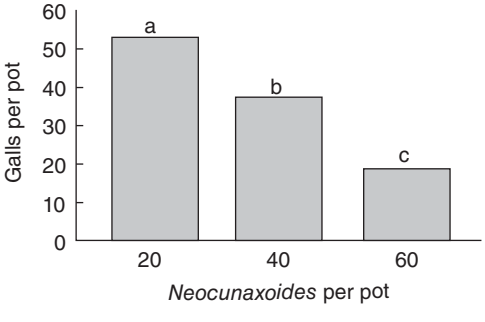

Fig. 14.2. The effect of various numbers of *Neocunaxoides andrei* on the number of galls induced by *Meloidogyne javanica* on tomato roots in pots (based on data in Table 3, Shoala and El Kady, 2009).

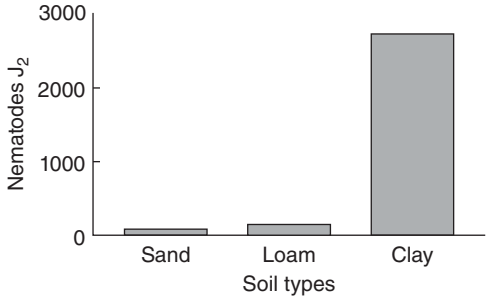

Fig. 14.3. The effect of three soil types on the number of surviving J_2 of *Meloidogyne javanica* when challenged by *Tyrophagus putrescentiae* (based on Table 1B in Walia and Mathur, 1997).

ratios than detrivores and fungivores, indicating feeding at several trophic levels.

Nematophagous mites occur in very large numbers in various soils. In Germany, about 37,500 nematophagous Acari/m^2 were estimated to occur below a soil depth of 15 cm, the numerical value for compost being tenfold (Karg, 1983). The presence of such large numbers of predators in the soil suggested that under suitable conditions they might protect field crops from nematode damage. El-Banhawy *et al.* (2006) found *T. semipenetrans* everywhere in the soils of Egyptian citrus groves. These authors noted that in localities with large numbers of mesostigmatid mites, nematode infestations were negligible, suggesting a control effect of the mites. Further support for this hypothesis was given by Elkins and Whitford (1982), who showed that predatory Acari regulated the numbers of non-pest nematodes in a desert ecosystem. Walter and Ikonen (1989) postulated that in systems where most primary production occurs below ground (i.e. grasslands), where nematode biomass is correlated with plant root biomass and where nematode densities are highest in the root area (rhizosphere), nematophagous arthropods (including mites) could be important predators of plant-feeding nematodes. In addition, tardigrades, which are efficient nematode predators, are in turn consumed by predatory arthropods, including mites. This association led Hyvönen and Persson (1996) to conclude that the impact of arthropod predators on nematode regulation was greater than it seemed when based only on nematode numbers. McSorley and Wang (2009) noted the relative but consistent suppression of root-knot nematodes (*Meloidogyne* spp.) concomitant with the occurrence of invertebrate predators, especially spring-tails (Collembola) and mites. Collectively, these data indicate that under certain conditions the populations of phytonematodes may be naturally reduced by mites; increasing and promoting such reductions could thus be a viable option. In none of the observations and experiments (with the exception of El Banhawy *et al*., 1997, who reported only little improvements in citrus fruit quality) any effect in population reduction of nematode and quantity and quality of the crop was observed. The economic dimension of these data thus remains obscure.

Notwithstanding all the above, estimates of predation rates in the field may be too high, feeding on nematodes in the soil being probably much lower than in the laboratory, as the soil provides nematodes refuge ('enemy free space') from their predators (Stirling, 1991). Phytonematodes are a normal component of functioning soil ecosystems, becoming pests when they are no longer constrained by the buffering mechanisms that usually keep them in check (Stirling, 2011). Due to agricultural practices such mechanisms usually do not exert their buffering effects in the soil, and therefore other means of control must be applied.

14.5 Augmenting and Conserving Mites for Phytonematode Control

14.5.1 Prospecting for nematophagous mites

Many authors reported that nematophagous predators abound in natural and agricultural soils. Walter and Kaplan (1990), who collected mites associated with citrus roots in Florida, suggested that nematode BC could be accomplished by large numbers of species with low feeding rates. Acari that are rare in the field but possess rapid development rates and high reproductive outputs are the most likely source for suitable predators. According to

El-Banhawy *et al.* (2006), light to medium soils of citrus groves carry an abundance of mesostigmatid predators, which would therefore be optimal sites to search for suitable nematophages. Of 63 mesostigmatid species obtained from grassland soils in Colorado, 54 (85%) produced viable progeny with only nematodes as food (Walter and Ikonen, 1989). On another continent, Beaulieu and Walter (2007) collected almost 70 mesostigmatic mites from the soil and litter of Australian forests, most of which were generalist predators that readily fed on nematodes. Predatory Mesostigmata were much more abundant in soils treated with long-term organic fertilizers, as compared to soils treated with chemical fertilizers (Cao *et al*., 2011). Working in northern India, Walia and Mathur (1994a) collected mites and tested them for nematophagy. The suitable Prostigmata were abundant in upper soil layers, the Mesostigmata in deeper strata whereas the Cryptostigmata and Astigmata occurred in both layers. From southern Germany Buryn and Hartmann (1992) reported that the acarine soil fauna found under meadows was dominated by nematophagous Mesostigmata.

Acari were obtained from various soils in Japan and their potential nematophagy explored by offering them the bacterivorous nematode *Cephalobus* sp. (Muraoka and Ishibashi, 1976). A total of 40 nematode-feeding mites were thus obtained, of which 23 always fed on that diet, 12 'frequently' and the others 'occasionally'. These mites were members of 26 families, of which one was an astigmatid, 12 Mesostigmata and 13 Cryptostigmata. From these data Muraoka and Ishibashi (1976) speculated that the 'lower Cryptostigmata' (Oribatei Inferiorus) do not feed widely on nematodes, in contrast to the so-called 'higher Cryptostigmata' (Oribatei Superiores), especially members of the family Galumnidae.

In conclusion, numerous sites and methods are there to collect nematode-feeding mites, which can be obtained by various soil extraction methods (e.g. McSorley and Walter, 1991). A possible short-cut to attracting and recognizing the nematophagous mites could be by setting traps baited with ascarosides. The attracted mites (including members of the mesostigmatid family Macrochelidae, which feed on manure nematodes) may then be mass-reared, preferably on nematodes (Royce and Krantz, 1991). Their general life history parameters, preference and voracity for nematodes, along with other relevant parameters can then be assayed in the laboratory (Rockett, 1980; Abou-Awad *et al*., 2001; Heckmann *et al*., 2007; Castilho *et al*., 2009; and others). In addition, species of several common nematophages (e.g. *Gaeolaelaps*, *Macrocheles*), intended for use against insect pests, are commercially available (van Lenteren, 2012). The next step would be devising methods for their dispersal in the greenhouse and in the field.

14.5.2 Basic considerations

After the nematophagous mites have been obtained and mass-reared, several additional considerations come into play, such as the soil characteristics and its humidity, the traits of the nematophages, whether specialists or generalists (or of both predation modes, see below) and where and when to release them, nematode prey and the crop that is to be protected, as well as any other applied treatments (see Conservation, below). In general, light to medium humid soils are more suitable (El-Banhawy *et al*., 2006). By modifying the physical condition of soil, activity of the predators can be increased by increasing their impact on the nematodes (Stirling, 1991). Small, flattened, narrow and elongate mites with a flexible opisthosoma are better adapted to movement in the soil. They move easier and faster through minute soil channels and pores, providing the mites with better access to nematodes, possibly even in deeper soil strata (Walter and Kaplan, 1990). On the other hand, many species of *Lasioseius*, known to feed on nematodes, seldom occur in deep soil strata due to their relatively large size. Thus despite having higher oviposition rates when feeding on nematodes (e.g. Britto *et al*., 2012), the impact of *Lasioseius* on pest nematodes in the soil may be limited. Amin *et al.* (1999) released *L. athiasae* and *Protogamasellus discorus* Manson 24, 48 and 96 h before inoculating

roots of bean plants with *M. javanica*. Highest reduction in nematode numbers was obtained when the predators were released 96 h prior to nematode inoculation. Shoala and El Kady (2009) released the predator *N. andrei* to tomato seedlings immediately after *M. javanica* was added. They obtained significant reductions in nematode galls. Timing of predator release may thus depend on the crop to be protected.

The host plant (the particular crop) attracts its specific nematode pests and affects their fecundity. Tomato, cabbage and maize (corn) support abundant nematode numbers, whereas sorghum, pepper and cotton are poor hosts thus also have smaller numbers of their predators (Kerry, 1997). In grassland systems, where nematode densities are highest in the rhizosphere, nematophages seem to be associated with the root systems and could be important nematode predators (Walter and Ikonen, 1989).

14.5.3 Specialist versus generalist acarine predators

As noted, several species in the families Eviphididae, Alicorhagiidae and Bimichaelidae feed exclusively on nematodes. None, however, has so far been accredited with affecting pest nematode populations. Nevertheless, their specialization, reflected by actively foraging for nematode prey, may render them suitable for rapidly reducing large nematode numbers at certain 'hot spots', especially in the rhizosphere of crops particularly susceptible to nematodes. However, such predators will be disadvantageous when nematode numbers are low. Generally they attack nematodes mostly when encountering them in the soil, but can feed on other prey (including fungi) during periods of nematode scarcity (Stirling, 1991). Symondson *et al.* (2002) concluded from field studies that in more than 75% of cases, generalist predators (of all pest groups), whether single species or species assemblages, reduced pest numbers significantly. Members of either functional group may therefore be suitable for different situations.

14.5.4 Should one or more nematophagous mites be applied?

Various authors have claimed that diverse predator assemblages can be more effective in controlling prey populations than a single enemy, whereas others have shown no effect of predator diversity on prey mortality, or even negative effects (e.g. due to intra-guild predation and/or interference). Tylianakis and Romo (2010) reviewed this question and suggested that natural enemy diversity may promote the biological control of arthropods only in heterogeneous systems. No data are available related to nematophagy by mites, but any relevant information could be very useful.

Notwithstanding the lack of relevant data, several nematophagous mites, whether mostly sessile or active hunters for nematodes in different soil strata, and whether generalists or specialists, could probably be tried together in augmentation efforts.

14.5.5 Resource competition

As noted, most nematophagous soil mites are omnivores, and the availability of fungi, other small prey and even their own smaller stages could detract from their nematophagy (Walia and Mathur, 1994b; Wiethoff *et al*., 2004). The risk that generalist predators will consume alternate prey instead of the target pest, and thus reduce their pest-controlling effect, was explored by Koss and Snyder (2005). A related problem could be the interference of certain mites in the BC of other pests, by preying on entomopathogenic nematodes (EPN) (Epsky *et al.*, 1988). A recent example is the acarid *Sancassania polyphyllae* (Zachvatkin), which attacks the infective nematode juveniles (IJs) of the EPN *Steinernema glaseri* (Steiner) emerging from the cadavers of their beetle host (Cakmak *et al*., 2013).

14.5.6 Conservation

The above text has dealt mostly with the augmentation of nematophagous mites, but the

populations of these mites can also be conserved by various means. Koehler (1997), referring only to predatory Gamasina (Mesostigmata) in agroecosystems, reported that tillage techniques, such as ploughing, reduce the abundance and diversity of these mites, as does soil compaction by machinery. On the other hand, no-tillage practices, like mineral as well as organic fertilization, have positive effects on the mites. The application of pesticides, which must at times be used against various soil pests, including nematodes, may negatively affect the mites. Relevant information is therefore needed in order to conserve these predators. Carbofuran applied in Egyptian citrus orchards reduced the numbers of predatory soil mites and the populations of *T. semipenetrans* increased (El-Banhawy *et al.*, 1998). Aldicarb had a very detrimental effect on many Mesostigmata, especially on the deeper dwelling taxa, like the Rhodacaridae (Koehler, 1991). *B. dolichus* showed much sensitivity to abamectin (Chen *et al*., 2013). The chitin synthesis inhibitor triflumuron had no effect on soil micro-arthropods or nematodes, whereas the organophosphorus profenofos, the carbamate pyridaben and naphthalene decreased the numbers of soil arthropods (Xiong *et al.*, 2008). Fungicides had no effect on soil arthropods or on nematodes (Jaensch *et al.*, 2006). Guidelines advising plant protection personnel about the effects of pesticides on nematophagous mites (Smit *et al*., 2012) would contribute to the conservation of the predators.

14.6 Conclusion

In the previous two decades, several authors (Jatala, 1986; Stirling, 1991; Kerry, 1997) suggested the biological control of nematodes by various organisms, including Acari. The nematode-controlling effects of predators were often initially studied in pot experiments, providing important information, which seldom, if ever, reflect field conditions. The variations in soil cultivation and its conditions, as noted above, as well as the effects of the host plant and the present soil community, renders the application of *in vitro* results almost impossible. The reactions of the different predators to these variables (even if only one nematode pest is the target) add to the uncertainty. As noted above, their interactions are likely to affect the efficacy of the BC agent(s) and could account for lack of results under specific test conditions. Thus, the conclusion drawn from the research of the above noted scientists was that nematophagous mites, alone or with other predators, will not provide long-term, economic phytonematode control. Several approaches should therefore be tried in various combinations in order to promote integrated nematode control. These could include agrotechnical methods, such as soil aeration and solarization, adding soil amendments (Minor and Norton, 2004), green manure such as Asteraceae (Oka, 2010, 2012), crop rotation, resistant and tolerant cultivars, as well as chemicals ('soft' pesticides) and natural enemies.

Kerry (1997) emphasized the importance of basic knowledge about the nematode species and the ecology and biology of their natural enemies, and understanding the soil conditions in which they interact, as well as about the host plants. To which Jatala (1986) added the need for developing means for studying the dispersal patterns of nematodes and their enemies in the soil and their compatibility with agricultural chemicals. The collection of such data is an initial and essential step for devising control plans. Stirling (1991) suggested the need to obtain more accurate estimates of predation rates in the soil in order to arrive at better predictions of any predator's impact on pest nematode numbers. Such estimates should however take into account additional, sometimes competing, organisms (e.g. fungi, bacteria and other predators, including Collembola, rotifers and nematodes) that may cause nematode mortality in the soil.

Due to current environmental constraints in introducing exotic natural enemies, and especially generalists, the future of using exotic nematode-feeding mites is uncertain, unless specific predators can be found. The search for, research on and application of endemic nematophagous species appears at present to be a promising approach. However, information on numbers (or rates) of nematodes

killed will be useful when accompanied by data on increase in the quantity and quality of the crop.

Several nematodes, especially in the genus *Aphelenchoides*, occur in the buds and on the foliage of various plants, causing economic damage. As a group they are known as 'bud and leaf nematodes' (Christie, 1959). Although at times moving on to the surfaces of plants (e.g. *Aphelenchoides ritzemabosi* (Schwartz)), and thus exposed to natural enemies, their only recorded predator is the acarid soil mite *Rhizoglyphus echinopus* (Fumouze and Robin) (Sturhan and Hampel, 1977).

Finally, studies on the mite–nematode interactions have wider implications for the study of soil food webs and productivity. Biological indicators based on the abundance of soil organisms are powerful tools for inferring functional and diversity changes in soils affected by agricultural practices. Sánchez-Moreno *et al*. (2009) stated that bacterial-feeding and predatory nematodes, together with predatory mites, were abundantly present in organic no-till soils. These microorganisms were found associated with high values of organic matter and decomposition pathways and the prevalence of nematodes in the soil indicates long and complex soil food webs. Hence, nematode-based soil food web indices are useful indicators of other soil organisms such as mites, with similar functional roles and environmental sensitivities.

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Part V

Plant Growth-promoting Rhizobacteria

15 Plant Growth-promoting Rhizobacteria as Biocontrol Agents of Phytonematodes

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15.1 Introduction

Plant-parasitic nematodes (PPN) or phytonematodes are invertebrate obligate parasite of a large number of plants. There are about 197 genera containing 4300 species of phytonematodes. The important genera of PPN include: *Meloidogyne*, root-knot nematodes; *Pratylenchus*, lesion nematode; *Heterodera* and *Globodera*, cyst nematodes; *Tylenchulus*, citrus nematode; *Xiphinema*, dagger nematode; *Radopholus*, burrowing nematode; *Rotylenchulus*, reniform nematode; *Helicotylenchus*, spiral nematode; and *Belonolaimus*, sting nematode. Root-knot nematodes, *Meloidogyne* spp. have been found all over the world and are known to cause huge losses to crops of economic importance (Taylor and Sasser, 1978). About 90 species of root-knot nematode have been reported, but four of them, *Meloidogyne incognita*, *Meloidogyne hapla*, *Meloidogyne arenaria* and *Meloidogyne javanica*, cause most of the damage to crop plants (Taylor and Sasser, 1978). Other nematodes also cause damage to plants but loss varies from country to country depending upon the type of crop infested (Mathur *et al.*, 1986; Anonymous, 1987; Sasser, 1989; Adegbite, 2011).

Various management practices have been used all over the world to manage PPN and reduce the crop losses caused by these tiny creatures. The traditional methods of nematode management include regulatory, physical, cultural, chemical and biological. Each of these methods when used in isolation or in combination has either one or several limitations. For instance, soil solarization and hot water treatment of seeds and planting materials eliminate the nematode infection but at the same time it has been observed to harm the beneficial flora and fauna of the soil. Excessive use of agrochemicals (fertilizers and pesticides) poses serious threats to the environment as well as plant and human health. Likewise, regulatory methods check the introduction of nematode pests but cannot check the activity of nematode species already well established in an area of a country or the nematodes that spread by wind, water and insects. Therefore, new strategies or technologies need to be advocated to reduce the use of hazardous chemicals against phytonematodes without affecting quantity and quality of crop. One such alternative is the use of microorganisms as biocontrol agents against the target nematode pests (Garret, 1965; Jatala *et al*., 1979; Jatala, 1986; Nakkeeran *et al*., 2005; Wani, 2006, 2010; Wani and Wani, 2006; Mirik *et al*., 2008; Masoud and Abbas, 2009;

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 Biocontrol Agents of Phytonematodes (eds T.H. Askary and P.R.P. Martinelli)

Wani *et al*., 2009; Wani and Bhat, 2011; Mankua *et al*., 2012).

Several types of microorganisms have been used to improve nutrient availability in the soil (Freitas *et al*., 2007). The plant rhizosphere interacts with a myriad of microorganisms that colonize in, on and around the roots of growing plants. Numerous beneficial microorganisms are associated with root systems of higher plants (Khalid *et al*., 2006). They perform both beneficial and harmful associations with the plant roots. These specialized bacteria that inhabit plant roots and promote plant growth positively are called plant growth-promoting rhizobacteria (PGPR). Plant growth activity of several rhizobacteria has been determined by many workers (Arshad and Frankenberger, 1998; Zahir *et al*., 2004). Likewise, PGPR have been defined in different ways by different workers. Kloepper and Schroth (1978) defined PGPR as soil bacteria that colonize the roots of plants following inoculation on to seed that enhance plant growth. Vessey (2003) defined PGPR as a number of species of soil bacteria that inhabit the rhizosphere area of plants but which may grow in, on, or around plant tissues, and promote plant growth by a number of mechanisms. These bacteria rapidly colonize on to seeds or roots after inoculation in response to exudation and thus affect the growth of plants.

A considerable increase in sustainable agriculture has been noted by the use of PGPR in different parts of the world (Antoun and Prevost, 2006). Kloepper *et al.* (1980) revealed that PGPR are free-living bacteria that colonize plant roots, and after their application to seeds or crop plants, increased the growth and yield of plants and also reduced the damage caused by various pathogenic microorganisms. Bashan and Holguin (1998) divided PGPR into two classes, biocontrol-PGPB and PGPB, but this was not accepted widely by scientists. PGPR are generally divided into two types based on their existence in and around the plant roots. These are: (i) extracellular PGPR (ePGPR); and (ii) intracellular PGPR (iPGPR). The existence of ePGPR has been found in the rhizosphere, on the rhizoplane and in the spaces between cells of the root cortex, whereas the existence of iPGPR has been found inside the roots and in the specialized nodular structures of plant roots.

PGPR are catogorized as: (i) biofertilizers, increasing availability or uptake of nutrients to the plants; (ii) phytostimulators, producing plant growth hormones; (iii) rhizoremediators, degrading organic pollutants; and (iv) biopesticides, controlling diseases by the release of antipathogenic substances such as antibiotics or metabolites. Several workers have isolated different strains of bacteria and evaluated these bacterial strains for their plant growth-promoting activity and for the control of plant pathogens and crop pests (Becker *et al*., 1988; Bashan, 1998; Bertrand *et al*., 2001; Chakraborty *et al*., 2005). Various scientists and workers successfully used PGPR for increasing plant growth and yield of important crops of the world (Kloepper *et al*., 1980; Seldin *et al.* 1984; Zhang *et al*., 1996; Amara and Dahdoh, 1997; Chanway, 1998; Pan *et al*., 1999; Bin *et al*., 2000; Mariano and Kloepper, 2000; Asghar *et al*., 2002; Silva *et al*., 2006; Araújo, 2008; Figueiredo *et al*., 2008). Akhtar *et al*. (2012) in a comprehensive review on the effect of PGPR on pathogenic organisms demonstrated that plant growth-promoting substances can be utilized as biocontrol agents against several pathogens including nematode pests. Research workers have demonstrated different action mechanisms of PGPR in promoting plant growth (Glick, 1995; Gupta *et al*., 2000). The growth-promoting activity of PGPR and repression of pathogenic organisms may be due to increased solubilization of mineral nutrients and nitrogen fixation, uptake and availability of nutrients to the plant, by production of organic and inorganic compounds (antibiotics, by the production of growth hormones, and release of enzymes by the production of siderophores) and/or competition for nutrients by these microorganisms or by improving plant stress tolerance to drought, salinity and metal toxicity (Glick, 2004; Huang *et al*., 2004; Khan, 2005; Saleem *et al*., 2007; Ahmad and Hasnain, 2008; Ahmad *et al*., 2008; Kohler *et al*., 2009; Masoud and Abas, 2009).

The diverse array of PGPR that has been shown to facilitiate plant growth by various mechanisms include *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Bacillus*, *Bradyrhizobium*, *Pseudomonas*, *Rhizobium*, *Xanthomonas*, *Serratia*, *Streptomyces*, *Burkholderia* and many others. They are known to promote growth

through supression of plant diseases (bioprotectants), improvement in nutrient acquisition (biofertilizers) or phytohormone production (biostimulants) (Scher and Baker, 1982; Bashan and Holguin, 1998; Tenuta, 2003; Khalid *et al*., 2004, 2006; Siddiqui, 2006; Arshad *et al*., 2007, 2008; Gravel *et al*., 2007; Contesto *et al*., 2008; Mubeen *et al*., 2008; Stamford *et al*., 2008; Naiman *et al*., 2009; Poupin *et al*., 2013). Since PGPR play an important role in plant growth promotion, scientists have shown much interest in their commercialization so as to exploit them both as biofertilizer and biopesticide for various crops. Numerous reviews have been written by researchers from time to time describing various aspects of PGPR but my aim in this chapter is to discuss the vital role of PGPR as biological control agent of phytonematodes. In this chapter various mechanisms involved by PGPR with respect to plant growth and nematode control have been described in detail.

15.2 Plant Growth-promoting Rhizobacteria (PGPR) Against Phytonematodes

The soil bacteria are one of the major groups of microbes in the rhizosphere of plants, ranging between 10^6 and 10^8 colony forming units (cfu) per gram (Clark, 1967). Several of these soil bacteria are known for their potential in improving plant health and act as biocontrol agents of phytonematodes (Stirling, 1991; Siddiqui and Mahmood, 1999).

Researchers have isolated and assessed various strains of rhizobacteria for plant-growth promotion and antagonistic activity against PPN and other pests (Becker *et al*., 1988; Chanway *et al*., 1989; Siddiqui and Mahmood, 1992, 1995a; Chanway and Holl, 1993; Glick *et al*., 1997; Jaizme-Vega *et al*., 1997; Bent *et al*., 2001; Cannayane and Rajendran, 2002). Prasad *et al*. (1972) and Prasad and Tilak (1972) were first to observe the toxic effect of *Bacillus thuringiensis* on eggs and juveniles of *M. javanica* under *in vitro* and natural conditions, respectively. The nematicidal effect of these bacteria have been reported against several PPN and against eggs and second stage juveniles (J_2) of root-knot nematodes (Rai and Rana, 1979; Zuckerman *et al*., 1993; Chen *et al*., 2000; Al Banna and Khyami-Horani, 2004). Various rhizosphere bacteria with antagonistic activity against PPN have been identified. Zavaleta-Meija and Van Gundy (1982) found that rhizobacteria have biocontrol activity towards root-knot nematodes and they showed that more than 12% of the rhizobacteria tested brought a reduction in the number of galls of *M. incognita* on cucumber and tomato. Oostendorp and Sikora (1989) reported the beneficial interaction among nematodes, PGPR and microbes. Sikora (1992) also reported that 7–10% of the bacteria isolated from rhizosphere of potato, sugarbeet or tomato root systems showed antagonistic activity against cyst and root-knot nematodes. Sikora and Hoffmann-Hergarten (1993) revealed that plant health-promoting rhizobacteria (PHPR) influence the intimate relationship between the PPN and its host by regulation of nematode behaviour during the early root penetration phase of parasitism, which is extremely important for crop yield. Sikora (1988) observed that *Bacillus subtilis* have been found effective in controlling *M. incognita*, *M. arenaria* and *Rotylenchulus reniformis* on cotton, sugarbeet and groundnut, respectively. Strains of *Pseudomonas chitinolytica* were also shown to reduce *M. javanica* on tomato as observed by Spiegel *et al*. (1991). Smith (1994) reported that a strain of *Bacillus* sp. (23a) reduced *M. javanica* densities on tomato and a strain of *Pseudomonas fluorescens* (PF1) reduced the number of galls and egg masses of *M. incognita* on roots of tomato (Santhi and Sivakumar, 1995). Toxicity of *Bacillus* spp. and other rhizobacteria for antagonistic activity against nematodes and for plant growth improvements have also been observed by various workers (Becker *et al*., 1988; Chanway *et al*., 1989; Bent *et al*., 2001). Chanway (1995) used *Bacillus polymyxa* strain L6-16R to increase seedling emergence of western hemlock after seed bacterization with the bacterial strain.

PGPR have been found effective as a biocontrol agent against root-knot nematodes, *Meloidogyne* spp. (Becker *et al*., 1988; Sikora, 1988; Hallmann *et al*., 1997). Zavaleta-Mejia and Van Gundy (1982) assessed 244 PGPR isolates and observed that only 125 isolates of PGPR have potential to control *M. incognita* on tomato

and cucumber. Several other workers also revealed that PGPR, besides acting as biocontrol agents against phytonematodes, improved growth and yield of crop (Suslow and Schroth, 1982; Hallmann *et al.*, 1997, 1998; Kloepper, 1995; Hoffmann-Hergarten *et al*., 1998; Hallman, 2001). Many isolates of *Agrobacterium radiobacter*, *B. subtilis*, *Pseudomonas* spp. that are known for antibacterial and antifungal activity have been found as potential biocontrol agents against phytonematodes. PGPR, *Bacillus cereus* strain S18 is considered an effective biocontrol agent against nematodes, particularly cyst and root-knot species (Oostendorp and Sikora, 1986, 1989, 1990; Sikora and Hoffmann-Hergarten, 1993). In naturally infested field soil, the nematicidal activity of bacteria reduced nematode densities by 50–100%. The potential of several strains of PGPR as biocontrol agents has been reported by several other researchers on different crops (Stirling, 1984; Weller, 1988; Smith, 1994; Hallmann *et al*., 1995, 1997; Hoffmann-Hergarten *et al*., 1998; Siddiqui and Mahmood, 1999; Reitz *et al*., 2000). Enhanced growth of wheat and soybean plants inoculated with *Azospirillum brasilense* have been reported by Bashan *et al*. (1990). Species of *Bacillus* which showed nematicidal effect and caused 100% juvenile mortality against root-knot nematode, *M. incognita* and cyst nematode, *Heterodera cajani*, *Heterodera zeae* and *Heterodera avenae* include *B. subtilis*, *Bacillus pumilus* (Gokte and Swarup, 1988), *B. cereus* (Kempster *et al*., 2001) and *Bacillus licheniformis* (Siddiqui and Mahmood, 1992). Nakayama *et al*. (1999) were the first to report about the broad spectrum activity of antibiotics by PGPR from culture filtrates or purified antibiotics. Seed treatment with antagonistic rhizobacteria reduced the early infection on sugarbeet caused by *Heterodera schachtii* (Oostendorp and Sikora, 1986). A considerable inhibition in nematode multiplication has been observed by Becker *et al.* (1988) in clover plants treated with *B. licheniformis* than in chickpea plants treated with *Alcaligenes faecalis*. Field experiments on the effect of PGPR revealed that *B. thuringiensis* var. *kurstaki* h3 was highly effective against *Radopholus similis* in banana (Mena *et al*., 1997). Carneiro *et al.* (1998) investigated 21 strains of *Bacillus* spp. and found they were effective against J_2 of *M. javanica*. It was noted that bacterial supernatant and entire culture of *B. thuringiensis*, *Bacillus laterosporus* and *A. brasilense* resulted in the highest mortality of freshly hatched J_2 within 24–48 h, however in different treatments of *B. thuringiensis*, *Bacillus aizawai*, *Bacillus morrisoni* and *Bacillus circulans*, nematodes were immobilized only. Greenhouse bioassays confirmed the nematicidal effect of rhizobacteria under *in vitro* tests. The effect of treatments may be due to presence of extracellular toxins with strong nematicidal activity. On the basis of an experiment Siddiqui and Mahmood (1998) revealed that combined application of PGPR, *P. fluorescens* and AM fungi, *Glomus mosseae* under different soil types caused higher reduction in galling, soil population of nematodes and morphometrics, than their individual application on tomato infested with root-knot nematodes. Siddiqui *et al*. (1998) observed that *P. fluorescens* in combination with arbuscular mycorrhizal fungi (AMF) and *Paecilomyces lilacinus* act as an effective biocontrol agent against cyst nematode, *H. cajani.* The nematicidal activity of *P. fluorescens* and *B. thuringiensis* is known against juveniles and adults of *M. incognita* infecting tomato plants (Hanna *et al*., 1999). Jonathan *et al.* (2000) reported that application of *P. fluorescens* and *Bacillus* spp. resulted in profuse root development in banana, tomato, clover plants and betel vine and decreased populations of *M. incognita*. However, *P. fluorescens* and *Bacillus* spp. were found most effective against *M. incognita* in clover; whereas fewer galls and larger roots were observed in plants treated with these bacteria. Rajendran *et al.* (2001) observed that for the management of *M. incognita* and *Tylenchus semipenetrans* in horticultural crops such as citrus, tomato, potato and chilli, *P. fluorescens*, *Bacillus* spp. and vesicular arbuscular mycorrhiza (VAM) could be used successfully as biocontrol agents. In a similar study, Siddiqui and Mahmood (2001a) observed significant reduction in root galling and nematode multiplication and improvement in plant growth of chickpea by the use of PGPR, *P. fluorescens*, *Azotobacter chroococcum* and *A. brasilense*, in combination with root symbionts, *Rhizobium* and *G. mosseae*. Siddiqui *et al*. (2001) found two strains of *P. fluorescens* (GRP3 and PRS9) alone and in combination with some organic manures and inorganic

fertilizers, improved plant growth parameters of tomato and at the same time reduced root galling and multiplication of nematodes. However, *P. fluorescens* GRP3 in combination with organic manure proved most effective in the management of *M. incognita* on tomato. A talc formulation of *Bacillus* spp. when applied against root-knot nematode resulted in significant reduction in the number of root galls and caused improvement in plant growth parameters of tomato, pepper and banana (Kokalis-Burelle *et al*., 2002; Jonathan and Umamaheshwari, 2006).

Endophytic bacteria such as *Brevundimonas vesicularis*, *Serratia marcescens*, *Burkholderia cepacia*, *Phyllobacterium rubiacearum*, *Pseudomonas aeruginosa* and *Rhizobium etli* have the capability of colonizing internal host tissues of the plant, making them a valuable tool in improving crop growth and host's response against nematodes such as *M. javanica*, *M. incognita* and *Globodera* sp. (Sikora and Hoffmann-Hergarten, 1993; Hallmann *et al*., 1995, 1998, 2001; Mahaffee *et al*., 1997; Hasky-Gunther *et al*., 1998; Siddiqui *et al*., 1998; Jonathan *et al*., 2000; Munif *et al*., 2000; Siddiqui and Shaukat, 2002; Nakkeeran *et al*., 2006). Endophytic bacteria are known to colonize roots of plants and improve their growth and thus have a potential to develop as biocontrol agents of PPN (Siddiqui and Shaukat, 2003b). It is further suggested that these bacteria can be developed into biological control agents as they are easy to culture and can be applied as seed treatment, are capable of reducing the initial root damage, can escape microbial competition and do not produce any phytotoxic symptoms on plants, instead they assist in plant growth promotion and depend on root exudates for multiplication and for their antagonistic activities. Munif *et al*. (2000) demonstrated biocontrol activity of endophytic bacteria against *M. incognita* in tomato. Researchers have also revealed that *Pseudomonas aeruginosa* brought about suppression of root-knot nematode on tomato and improved plant growth (Siddiqui and Ehteshamul-Haque, 2000a,b, 2001; Siddiqui *et al*., 2002a,b; Siddiqui and Shaukat, 2003a). Likewise, Siddiqui *et al*. (2001) reported control of chickpea root-knot nematode with *P. aeruginosa*. Dhawan *et al*. (2004) revealed that J_2 of *M. incognita* after 24 h exposure to standard filtrates (S) and S/10 dilution in four native strains of *B. thuringiensis* caused complete death of *M. incognita* juveniles. The endophytic bacterial isolates of *B. subtilis* (EPB 5, 22, 31 and EPC 16) prepared in talc-based formulations were evaluated for their biocontrol potential against nematodes of banana, *M. incognita*, *Pratylenchus coffeae*, *R. similis* and *Helicotylenchus multicinctus*, under pot culture conditions (Jonathan and Umamaheshwari, 2006). The combined treatment of EPB 5 brought about significant improvement in plant growth parameters and decreased soil population of nematodes. Siddiqui (2004) assessed the influence of *P. fluorescens*, *A. brasilense*, *A. chroococcum* and composted organic fertilizers alone and in combination on tomato seedlings inoculated with *M. incognita*. The treatments resulted in reducing the root galls and improving the plant growth parameters. The best result was obtained with *P. fluorescens* followed by *A. chroococcum* and *A. brasilense*. However, the best improvement in effect was observed when bacteria were used in combination with composted organic fertilizers like cow dung, goat dung and poultry manure. *A. brasilense* inoculated singly or in combination with AMF, *Glomus claroideum* favoured papaya growth during the nursery phase (Alarcon *et al*., 2002). Jaizme-Vega *et al*. (2004) tested three strains of PGPR, *B. consortium* and AMF against *Meloidogyne* sp. infesting papaya and found a decrease in nematode population and increase in plant growth. The same positive effects have been observed in other tropical crops inoculated with PGPR (Jaizme-Vega *et al*., 1997, 2004). Nakkeeran *et al*. (2005) are of the opinion that there is large scope for developing biopesticide formulations from various strains of PGPR at the commercial level. *Brevibacillus brevis* and *B. subtilis* were found to have strong nematicidal effect, killing J_2 of *M. javanica* (Li *et al*., 2005). Siddiqui and Singh (2005) observed improvement in plant growth and reduction in galling and nematode multiplication by the application of bacteria *P. striata* and *Rhizobium* sp. on pea plants. Other strains of *Bradyrhizobium*, *Pseudomonas*, *Bacillus*, *Azospirillum*, *Azotobacter*, *Enterobacter* and *Serratia* have also been described as plant growth-promoting substances with good results in terms of plant

growth promotion, yield as well as nematode control (Burr *et al*., 1978; Burr and Caesar, 1984; Caesar and Burr, 1987; Polonenko *et al*., 1987; Kloepper, 1993, 1995; Dobbelaere *et al*., 1999; Burkett-Cadena *et al*., 2008). The reduction in root galling and production of egg masses caused by root-knot nematodes has been reported with the application of talc formulation of *P. fluorescens* and *B. thuringiensis* on crop plants (Verma *et al*., 1999; Khyami-Horani and Al-Banna, 2006). The reduction in infestation of *M. incognita* by application of *Bacillus* spp. has also been observed by other research workers (Oostendrop and Sikora, 1990; Sikora, 1992; Sikora and Hoffmann-Hergarten, 1992). Talc formulations prepared from isolates of *Bacillus* sp. and *Micrococcus* sp. were found potentially effective against *M. incognita* (Linh, 2008). PGPR with wide scope for commercialization include some species of *Pseudomonas* such as *P. putida* and *P. aeruginosa*, *B. subtilis* and other *Bacillus* spp., *Bradyrhizobium*, *Azospirillum*, *Azotobacter*, *Enterobacter* and *Serratia* (Burkett-Cadena *et al*., 2008). The potential PGPR isolates are formulated using different organic and inorganic carriers through solid or liquid fermentation technologies. Burkett-Cadena *et al*. (2008) tested commercially available rhizobial inoculants (Equity, Bio-Yield and AgBlend) and FZB42 strain for growth promotion in tomato and pepper. Significant reduction in nematode eggs per gram root, juveniles per millilitre of soil and galls per plant on tomato was recorded by the inoculants Equity (multiple strains), BioYield (two strains), FZB42 and AgBlend, containing microbial metabolites. In the case of FZB42, active colonization of rhizosphere was found resulting in nematode suppression. It was further observed that induction of suppressive soil against *M. incognita* was related to bacterial population size in the rhizosphere. Siddiqui and Akhtar (2007) revealed that *P. aeruginosa* individually and in combination with other micoorganisms, *Aspergillus awamori* and *Glomus intraradices*, caused greatest increase in plant growth of chickpea and suppressed root galling and nematode multiplication caused by *M. incognita*. Siddiqui *et al*. (2007) observed that the highest inhibitory effect on egg hatching and root penetration of *M. javanica* was observed in *P. putida* followed by *Pseudomonas alcaligenes*, *Paenibacillus polymyxa* and *P. pumilus*. PGPR species when inoculated alone or together with *Rhizobium* increased plant growth, both in *M. javanica*-inoculated and *M. javanica*-uninoculated plants. Among PGPR, *P. putida* caused greater improvement in plant growth parameters and higher reduction in root galling and nematode multiplication followed by *P. alcaligenes*, *P. polymyxa* and *P. pumilus*. The combined use of *Rhizobium* with PGPR brought higher reduction in galling and nematode multiplication than their individual inoculation. A significant increase in plant growth characters and reduction in root galling and nematode multiplication has been observed due to individual and combined applications of *G. intraradices*, *P. putida* and *P. polymyxa* on pea seedlings. But combined inoculation of *G. intraradices*, *P. putida* and *P. polymyxa* were found more effective in reducing the root galling and nematode multiplication than individual inoculations (Siddiqui and Akhtar, 2007). Akhtar and Siddiqui (2007) observed that inoculation of plant with *G. intraradices*, *P. striata* and *Rhizobium* sp. caused significant increase in plant growth, number of pods, chlorophyll, phosphorus and potassium content in nematode-inoculated plants. However, greater improvement in plant growth characters and reduction in root galling and nematode multiplication was observed in *Rhizobium*-inoculated plants followed by *P. striata* and *G. intraradices*. The maximum reduction in root galling and nematode multiplication was observed by the combined application of *G. intraradices*, *P. striata* and *Rhizobium* sp. Some isolates of *Bacillus* species have also been found to have good potential as biocontrol agents against PPN (Siddiqui and Shakeel, 2007). Linh (2008) evaluated the nematicidal effect of nine strains/isolates of *Bacillus* sp. and *Micrococcus* sp. against *M. incognita* infesting tomato. It was observed that all the nine strains/isolates showed nematicidal activity against *M. incognita* and increased seed germination and plant growth of tomato under *in vitro* and *in vivo* conditions. Some strains of *Bacillus* and *Micrococcus* brought about significant increase in root and shoot length and dry matter production while certain others caused a decrease in these variables of plant growth parameters. This improvement in plant growth parameters may

be due to increase in the release of antibiotics, inhibition of early penetration of roots by PPN and alteration of root exudates that possibly contain polysaccharides and amino-acids that modify behaviour of nematodes. Siddiqui and Akhtar (2008) in another study observed that combined use of neem litter with PGPR, *P. putida* + *G. intraradices* proved better in reducing root galling and nematode multiplication and thereby improving plant growth of tomato.

Akhtar and Siddiqui (2008a) evaluated the effect of *G. intraradices*, *P. alcaligenes* and *B. pumilus* on the root-rot disease complex caused by the root-knot nematode *M. incognita* and the root-rot fungus *Macrophomina phaseolina* in chickpea. They observed greater increase in dry weight of shoot, pod number, chlorophyll, nitrogen, phosphorus and potassium content in plants inoculated with *P. alcaligenes* followed by plants inoculated with *G. intraradices* or *B. pumilus*. However, *G. intraradices*, *P. alcaligenes* and *B. pumilus* when inoculated combined caused greater increase in the above plant growth parameters than *P. alcaligenes* + *B. pumilus* or *G. intraradices* + *B. pumilus*. In another study, the isolates of *P. putida* and *P. alcaligenes* and parasitic fungi *Pochonia chlamydosporia* and *P. lilacinus* in combination with *Rhizobium* were found effective in reducing root galls and multiplication of *M. javanica* (Siddiqui and Akhtar, 2009b). *P. putida* proved more effective than *P. alcaligenes*. Akhtar and Siddiqui (2008b) used PGPR, *Pseudomonas* and *Rhizobium* isolates for the management of root-rot complex disease involving phytonematodes and pathogenic fungi on chickpea and received encouraging results in terms of nematode control and plant growth. Likewise, Siddiqui and Akhtar (2009a) observed significant increase in plant growth of tomato and reduction in root galling due to application of antagonistic fungi and PGPR, *B. subtilis*, *P. polymyxa* and *B. cepacia*. *P. polymyxa* when used in combination with *P. lilacinus* or *P. chlamydosporia* resulted in greater improvement in plant growth characters and reduction in root galling than when used alone. Siddiqui and Akhtar (2009a,b) observed that *P. putida* (MTCC No. 3604), *P. alcaligenes* (MTCC No. 493) and *Pseudomonas* isolate (Ps28) alone and in combination with fungi *P. lilacinus* or *Rhizobium* sp. caused reduction in root galling, nematode multiplication, root penetration of juveniles and plant growth improvement in tomato and chickpea. However, greatest improvement in plant growth was observed when *Rhizobium* was inoculated in combination with fungus or PGPR. Bhat and Wani (2012) revealed in their studies that PGPR alone and in combination with *P. lilacinus* significantly improved the growth of black gram and caused reduction in root galling caused by *M. incognita*. The reduction in nematode population was observed by Siddiqui and Futai (2009) by the application of *P. putida* with cattle manure. Abuzar and Haseeb (2010) studied the effect of soil application of two rhizobacteria (MTCC and Pf-5) and urea treatments separately and in combination on free-living and plant-parasitic nematodes. They revealed that all the treatments significantly decreased the population of nematodes and increased plant growth characters of pigeonpea. However, the combined treatments were more effective than individual applications. Wani *et al.* (2010) observed improvement in growth characters of pine seedlings when inoculated with *Bacillus* sp. Isolates of *Pseudomonas* and *Bacillus* have been found effective as biocontrol agents against PPN (Zuckerman *et al*., 1993; Siddiqui *et al*., 2009; Elyousr *et al*., 2010). Anwar-ul-Haq *et al*. (2011) observed that plants treated with *P. fluorescens*, *P. putida*, *Bacillus* spp. and *Azotobacter* spp. caused improvement in plant growth of tomato and suppression in root galls caused by *Meloidogyne* spp. Shankar *et al*. (2011) assessed the antagonistic effect of *P. aeruginosa* on *M. incognita*-infested tomato plants and observed that isolates I and II of *P. aeruginosa* resulted in better plant growth as compared to un-inoculated ones.

Plant growth-promoting substances have been observed to induce systemic resistance in plants against nematode and other pests. Several workers have used strains of PGPR for inducing systemic resistance in plants against nematode pests (Sikora and Hoffmann-Hergarten, 1992). Sikora (1988) observed that *B. subtilis* induced resistance against *M. incognita* and *M. arenaria* in cotton. *P. fluorescens* has been found to induce systemic resistance and inhibit early root penetration of cyst nematode,

H. schachtii, on sugarbeet (Oostendrop and Sikora, 1990). Santhi and Sivakumar (1995) observed that root-knot infestation level caused by *M. incognita* on tomato was reduced due to root dip treatment with *P. fluorescens* strain Pf1. Similarly, reduction in root-knot infection caused by nematodes in tomato has been observed by the application of *P. chitinolytica* (Spiegel *et al*., 1991). Treatment of rice seed with PGPR separately and in combination with chitin and neem cake resulted in significant reduction in the population of rice root nematode *Hirschmanniella oryzae* (Swarnakumari and Lakshmanan, 1999; Swarnakumari *et al*., 1999). PGPR-mediated induced resistance in some dicot (bean, cucumber, tobacco and tomato) and monocot (rice, maize and sugarcane) plants has been reported by many workers (Swarnakumari, 1996; Schneider *et al*., 1996; Sung and Chung, 1997; Van Loon *et al*., 1998; Munif *et al*., 2001). Soil application of *P. fluorescens* reduced the soil population of root nematodes *R. similis*, *P. coffeae* and *H. multicinctus* at the same level as carbofuran (Shanthi *et al*., 2003). Fluorescent products of *Pseudomonas* were found to have inhibitory effects on hatching and penetration of nematodes and colonization on pigeonpea root (Siddiqui *et al*., 2005).

Pseudomonas and *Bacillus* are well known for their antagonistic effects and their ability to trigger induced systemic resistance (ISR). Direct antagonism has been observed by many species of *Bacillus* against nematodes and other pests (Handelsman *et al*., 1990; Liu and Sinclair, 1992; Yu *et al*., 2002); elicited ISR results in reduction in disease severity and plant growth improvement (Kloepper *et al*., 2004). In banana, *P. fluorescens* and *B. subtilis* were found to trigger ISR against lesion nematodes (Shanthi and Rajendran, 2006). The antagonistic activities of PGPR might be due to synthesis of hydrolytic enzymes (Van Loon, 2000; Neeraja *et al*., 2010; Maksimov *et al*., 2011), competition for nutrients and suitable colonization of niches at the root surface (Kamilova *et al*., 2005; Van Loon, 2007). The regulation of ethylene level through the ACC-deaminase enzyme can act to modulate levels of ethylene in response to stress imposed by the infection (Van Loon, 2007) and production of siderophores and antibiotics (Riley, 1993; Riley and Wertz, 2000; Andrews *et al*., 2003; Dimkpa *et al*., 2009; Raaijmakers *et al*., 2010; Maksimov *et al*., 2011). Beneduzi *et al*. (2012) reported that rhizobacteria-induced systemic resistance in plants resembles pathogen-induced systemic acquired resistance (SAR), which can make uninfected plants more resistant. Rahanandeh *et al.* (2012) isolated and tested eight strains of *Pseudomonas* from rhizosphere of a tea plantation and found seven strains which belonged to *P. fluorescens* caused 63% and one that belonged to *P. aeroginosa* caused 95% mortality of tea lesion nematode *Pratylenchus loosi*.

15.2.1 Mode of action

Plant-parasitic nematodes are attacked by a number of microorganisms present in the rhizosphere. These microorganisms in association with the plant rhizosphere have a profound effect on the nematode populations, influencing both the dynamics of nematodes and the dynamics of a large number of antagonistic organisms and parasites present in the rhizosphere (Kerry, 2000). Kerry (1980) in his study revealed that to obtain total control of the population of pathogenic organisms it is necessary to control their infective stages. PGPR are known to promote plant growth and are also involved in the management of PPN (Table 15.1). Biological control is achieved through several mechanisms such as parasitism, competition and antibiosis, which adversely affect the fitness, survival and multiplication of nematodes. The term antagonist is used for parasites, predators, pathogens, competitors and other organisms because they repel, kill and reduce, or inhibit PPN in the soil rhizosphere or at the aerial surface of the host plants. Many species of soil bacteria promote plant growth by producing phytohormones, by induction, uptake and mobilization of nutrients to plants, besides controlling soil-borne pathogenic microbes (Sinclair, 1989).

The variation in growth promotion due to inoculation of rhizobacteria is not uncommon and this is considered as a significant barrier for the assessment of these bacterial inoculants (Scroth and Weinhold, 1986). This

Table 15.1. Mode of action of PGPR for plant growth and nematode control.

PGPR	Plants	Plant parasitic nematodes	Mode of action	References
Bacillus thuringiensis	–	*Meloidogyne* sp.	Biocontrol (nematicidal property)	Prasad *et al*., 1972
Pseudomonas fluorescens, *P. putida*, *Pseudomonas* spp.	Potato, sugarbeet, canola and apple	–	Plant growth promoting	Burr *et al*., 1978; Suslow, 1980; Caesar and Burr, 1987; Lifshtiz *et al*., 1987
B. subtilis, *B. pumilus*	–	Root-knot and cyst nematodes	Biocide	Gokte and Swarup, 1988
Pseudomonas sp., *Bacillus* sp.	–	*Meloidogyne* spp.	Biocontrol (nematicidal)	Becker *et al*., 1988
Pseudomonas sp., *Bacillus* sp.	Sugarbeet	*Heterodera schachtii*	Biocontrol	Oostendorp and Skora, 1989,1990
Azospirillum brasilense	Wheat and soybean	–	Plant growth promoting	Bashan *et al*., 1990
B. penetrans, *P. chitinolytica*	Lentil, pea and other plants	*M. javanica*, *Meloidogyne* sp., plant-parasitic nematodes	Growth promoting/biocontrol	Stirling, 1984; Chanway *et al*., 1989; Oka *et al*., 1993
B. polymyxa	Spruce seedlings	Plant parasitic nematodes	Growth promoting/biocontrol	Chanway and Holl, 1993
P. fluorescens, *Rhizobium* sp.	Rice, cucurbits and other plants	Plant-parasitic nematodes, *Hirschmanniella oryzae*, *M. incognita*	Biocontrol	Kloepper and Scroth, 1978; Kloepper, 1993; Hallmann *et al*., 1995; Swarnakumari, 1996; Swarnakumari and Lakshmanan, 1999; Swarnakumari *et al*., 1999
Brevundimonas vesicularis, *Serratia marcescens*, *P. fluorescens*, *Burkholderia cepacia*, *Phyllobacter rubiacearum*	Cotton	*Meloidogyne incognita*	Colonize galled tissues and acts as biocontrol	Quadt-Hallmann *et al*., 1997; Hallmann *et al*., 1998, 1999
P. aeruginosa, *P. fluorescens*	Tomato, mung bean, soybean	*Meloidogyne javanica*, *M. incognita*	Root colonization and acts as biocontrol	Siddiqui and Ehteshamul-Haque, 2000a, 2001; Siddiqui and Shaukat, 2003a,b
Rhizobium etli, *P. fluorescens*	Beans, *Arabidopsis thaliana*, potatoes, cucumber	*M. incognita*	Decreased root galling and acts as biocontrol agent	Hallmann *et al*., 1998, 2001

Continued

Table 15.1. Continued.

PGPR	Plants	Plant parasitic nematodes	Mode of action	References
Rhizobacteria, *B. sphaericus*	Potato	*Globodera* sp.	Induce resistance/biocontrol	Hasky-Gunther *et al*., 1998
P. fluorescens, Enterobacter asburiae	Beans	–	Plant growth promoting	Mahafee *et al*., 1997
B. thuringiensis, *Bacillus* spp., *Rhizobium etli*, *B. cereus*	Brinjal, potato and other crops	*Meloidogyne* spp., *Globodera pallida*, *M. incognita*	Beta-exotoxin, nematicidal, lipopolysaccharides, biocontrol	Rai and Rana, 1979; Carneiro *et al*., 1998; Mahdy *et al*., 2000
Azospirillum spp., *B. thuringiensis*	Banana, tomato, pepper, cotton and wheat	Nematodes and *Radopholus similis*	Biocontrol	Mena *et al*., 1997; Bashan, 1998
A. brasiliense	Wheat	–	Auxin	Dobbelaere *et al*., 1999
B. thuringiensis, *Streptomyces* sp.	Lettuce	*M. hapla*	Biocontrol	Chen *et al*., 2000
Rhizobium sp.	Plants	–	Plant growth promoting	Biswas *et al*., 2000
Bacillus spp., *Rhizobium* sp.	Tomato and other plants	*M. incognita*, other root-knot nematodes	Biocontrol	Martinez-Ochoa, 2000; Munif *et al*., 2000
Bacillus spp.	Tomato, brinjal	*M. incognita*, *Meloidogyne* sp.	Systemic resistance, biocontrol	Weller, 1998; Munif *et al*., 2000
A. brasiliense	Carica papaya	–	Root and shoot growth	Alarcon *et al*., 2002
Pseudomonas sp.	–	Plant nematodes	*In vitro* nematicidal activity	Ali *et al*., 2002
P. fluorescens, *Pseudomonas* spp.	Mustard, soybean, tomato	Plant parasitic nematodes, *Rotylenchulus reniformis*	Auxin, nematicidal activity, induce resistance	Asghar *et al*., 2002; Niknam and Dhawan, 2002
Bacillus sp.	Soybean	–	Plant growth	Bai *et al*., 2003
P. fluorescens, *B. thuringiensis*	Pigeon pea	*Radopholus similis*, *P. coffeae* and *Helicotylenchus multicinctus*, *M. incognita*	Inhibitory on egg hatching and nematode multiplication	Shanthi *et al*., 2003; Dhawan *et al*., 2004
Bacillus spp., *Pseudomonas* spp.	Banana, potato	Plant parasitic nematodes, *Heterodera* sp.	Biocontrol	Aksoy and Mennan, 2004; Jaizme-Vega *et al*., 2004
Bacillus spp., *Pseudomonas* spp.	Wheat	–	Plant growth promoting	Khalid *et al*., 2004

P. aeruginosa, *Bacillus* sp., *Pseudomonas* sp.	Tomato, crop plants	*M. incognita*	Biocontrol	Siddiqui, 2004; From *et al*., 2005; Li *et al*., 2005
Pseudomonas sp., *Bacillus* sp.	Crop plants, *Arabidopsis*	–	ACC-deaminase	Balha *et al*., 2006; Contesto *et al*., 2006; Glick, 2006; Khalid *et al*., 2006
B. subtilis, *Pasteuria penetrans*	Banana, tomato	Plant parasitic nematodes	Biocontrol	Jonathan *et al*., 2000; Jonathan and Umamaheshwari, 2006
B. subtilis, *P. fluorescens*	Banana, grapevine	*Pratylenchus* sp., *M. incognita*	Induce systemic resistance, biocontrol	Shanthi *et al*., 1998, 2003; Shanthi and Rajandran, 2006; Van Loon, 2007
Rhizobium sp.	Lodgepole pine	–	Change in root hormone	Bent *et al*., 2001
P. putida	Tomato	–	Indole acetic acid (IAA)	Gravel *et al*., 2007
Rhizobium sp.	Wheat	Plant parasitic nematodes	Biocontrol	Afzal and Bano, 2008
Bacillus spp.	Potato, tomato, chilli	–	Auxins	Garcia de Salamone *et al*., 2001; Kamilova *et al*., 2005; Ahmad and Hasnain, 2008
Bacillus sp., *Pseudomonas* sp., *Burkholderia* sp., *Serratia* sp., *Enterobacter* sp.	Pea, wheat, chickpea	–	ACC-deaminase activity	Arshad *et al*., 2008
Rhizobium sp., *P. striata*	Wheat, chickpea	Plant parasitic nematodes	Biocontrol	Afzal and Bano, 2008; Akhtar and Siddiqui, 2008, 2009
Streptomyces acidiscabies	Cowpea	–	Hydroxamate siderophores production	Dimpka *et al*., 2008
Pseudomonas spp., *A. brasilense*, *A. chroococcum*, *Bacillus* spp., *Rhizobium* sp., *B. subtilis*	Tomato, pigeonpea, lentil, pea, chickpea	*Meloidogyne* spp., plant parasitic nematodes	Biocontrol, plant growth promoting	Siddiqui and Mahmood, 1995a,b, 2001b; Siddiqui, 2004; Siddiqui *et al*., 2005, 2009; Siddiqui and Shakeel, 2007; Siddiqui and Akhtar, 2008, 2009a; Siddiqui and Futai, 2009
Serratia marcescens, *P. fluorescens*, *Pseudomonas* spp., *Bacillus* spp.	Crop plants, tomato, chickpea	Plant parasitic nematodes, *M. incognita*, *Meloidogyne* spp.	Biocontrol, nematicidal activity	Siddiqui and Mahmood, 1997, 1999; Siddiqui, 2006; Mohamed *et al*., 2009; Elyousr *et al*., 2010

Continued

Table 15.1. Continued.

PGPR	Plants	Plant parasitic nematodes	Mode of action	References
Pseudomonas spp., *Bacillus* spp.	Plants	Plant parasitic nematodes and other pathogens	Release of hydrolytic enzymes, root colonization, production of antibiotics	Raaijmakers *et al*., 2010; Maksimov *et al*., 2011
Pseudomonas spp., *Bacillus* spp., *Azotobacter* sp.	Tomato	*M. incognita*	Plant growth, biocontrol	Anwar-ul-Haque *et al*., 2011
Pseudomonas spp., *Bacillus* spp., *Azotobacter* spp., *Burkholderia* sp., *Serratia* sp.	Crop plants, black gram	Plant parasitic nematodes, *M. incognita*	Biocontrol	Akhtar *et al*., 2012; Bhat and Wani, 2012
P. fluorescens, *P. aeruginosa*	Pigeonpea	*Pratylenchus loosi*	Biocontrol due to protease activity	Rahanandeh *et al*., 2012
Bacillus sp., *Rhizobacterium*, *Burkholderia phytofirmans*	Crop plants	–	Indole-3 acetic acid and cytokinin signalling	Chen *et al*., 2013; Zuniga *et al*., 2013
Bacillus sp., Rhizobacterium, Burkholderia phytofirmans	Crop plants	–	Degredation of Quorum sensing, IAA and cytokinin signalling	Ortiz-Castro *et al*., 2008; Chen *et al*., 2013; Zuniga *et al*., 2013

variability in plant growth response may be due to type of inoculant strain, quality of soil organic matter content, growing stage of plants, harvesting date of crop plants and growth parameters (Cakmakci *et al*., 2006). Some *Bacillus* and *Pseudomonas* spp. are known to produce an enzyme, ACC-deaminase, which hydrolyses the precursor of ethylene 1-aminocyclopropane,1-carbooxylic acid (ACC) and this may cause inhibition in root growth and ethylene concentration, thereby promoting plant growth (Penrose and Glick, 2001; Balha *et al*., 2006; Kohler *et al*., 2009). The influence of PGPR on the growth and development of plants may be direct or indirect. The indirect influence may be that they act as biocontrol agents of soil-borne pathogens and this indirect influence occurs when PGPR cause inhibition of the toxic effect of plant pathogens on plants by the release of inhibitory substances such as antibiotics, antifungal or antimicrobial metabolites, production of siderophores, cell wall-degrading enzymes like chitinases, cellulases and proteinases and by increasing the natural resistance of plants against plant pathogens (Sindhu *et al*., 1999; Jeun *et al*., 2004; Zahir *et al*., 2008; Narayanasamy, 2013). The direct effect of these rhizobacteria on PPN as demonstrated by some workers may be on egg hatching, nematode mobility and alteration in root exudates which prevents the attractiveness of nematodes towards the roots, reducing their penetration (Oostendorp and Sikora, 1990; Sikora and Hoffmann-Hergarten, 1992). In nature, especially in the rhizosphere area of plants, the most dominant and widely distributed genus has been *Bacillus* Cohn. The most important species of this genus in the rhizosphere soil are *B. subtilis*, *Bacillus mycoides*, *Bacillus pumilus*, *Bacillus megaterium*, *B. thuringiensis* and *Bacillus firmus* (Wipat and Harwood, 1999; Garbeva *et al*., 2003). These species of *Bacillus* are known to produce cytotoxins and other toxins (From *et al*., 2005), which are responsible for their toxicity against soil-borne pathogens (Zavaleta-Mejia and VanGundy, 1982). The effect of PGPR may be due to colonization of roots and biocontrol activity against nematodes and other pathogens (Benizri *et al*., 2001; Bloemberg and Lugtenberg, 2001).

Several workers have demonstrated the mode of action of *P. fluorescens* that brings changes in the root exudates which contain polysaccharides and amino acids. This prevents the early penetration of nematodes into the roots of host plants (Oostendrop and Sikora, 1990; Sikora, 1992; Sikora and Hoffmann-Hergarten, 1992). The significant reduction in root-knot infection caused by different species of *Meloidogyne* has been reported by Mahdy *et al*. (2000) in tomato plants. The direct antagonism by different species of *Bacillus* has been reported by many workers (Handelsman *et al*., 1990; Liu and Sinclair, 1992; Yu *et al*., 2002) and this may induce resistance in plants that results in reduction in disease severity caused by many plant pathogens (Kloepper *et al*., 2004). Shanthi and Rajendran (2006) reported that *P. fluorescens* and *B. subtilis* induce systemic resistance in banana plants against lesion nematode. Saharan and Nehra (2011) in a critical review discussed various PGPR, their mechanisms and multiple effects on various types of plants such as ornamentals, forest crops, vegetables, fruit crops and other agricultural crops. Beneduzi *et al.* (2012) demonstrated that rhizobacteria ISR in plants resembles pathogen-induced SAR that rendered uninfected plants more resistant to pathogens in many plant species. They further suggested that PGPR induce resistance through the salicylic acid-dependent SAR pathway, or require jasmonic acid and ethylene perception from the plant for ISR. Rhizobacteria, *Pseudomonas* and *Bacillus* species, are well known for their antagonistic effects and for their potential to trigger ISR. The antagonistic activities of PGPR might be due to synthesis of hydrolytic enzymes (Neeraja *et al*., 2010; Maksimov *et al*., 2011), competition for nutrients and suitable colonization of niches on the root surfaces (Kamilova *et al*., 2005; Maksimov *et al*., 2011). The regulation of plant ethylene level through ACC-deaminase enzyme can act to modulate the levels of ethylene in response to stress imposed by the infection (Van Loon, 2007), production of siderophores, antibiotics and phytohormones (Riley, 1993; Riley and Wertz, 2002; Andrews *et al*., 2003; Khalid *et al*., 2004, 2006; Dimkpa *et al*., 2009; Raaijmakers *et al*., 2010; Maksimov *et al*., 2011). Antagonistic activities of PGPR have also been found due to release of ACC-deaminase, degradation of Quorum sensing, indole-3 acetic acid and

cytokinin signalling (Ortiz-Castro *et al*., 2008; Chen *et al*., 2013; Zuniga *et al*., 2013).

15.3 Conclusions

From the above it is clear that PGPR plays an important role in the improvement of plant growth and management of phytonematodes, which are responsible for reducing the yield and quality of crops. The use of PGPR as biopesticide can act as an alternative to chemical pesticides that otherwise are hazardous for environment and human health. The role of several species of rhizobacteria such as *Bacillus*, *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, *Azospirillum*, *Azotobacter*, *Serratia*, *Streptomyces* and *Burkholderia*, in the biological control of phytonematodes has been discussed, which shows that they can play an important role in the growth of plants and biomanagement of phytonematodes by the colonization of plant root system and seed surface, uptake of nutrients, production of siderophores, fixation of atmospheric nitrogen, solubilization of minerals, release of phytohormones (auxins, cytokinin, gibberellins) and antibiotics. However, most of the work has been done in pots or under controlled conditions and therefore to ascertain the potentiality of PGPR, extensive studies are needed under field conditions. In recent years, some formulations of biopesticides from PGPR have been prepared that are available in the international markets, but more organized scientific research is needed to formulate new and effective biopesticide formulations from all the promising species and available isolates. The use of new formulations may be helpful in the biological control of PPN responsible for causing considerable economic losses to crop plants and can also be helpful in the development of sustainable agriculture.

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Part VI

Arbuscular Mycorrhizal Fungi

16 Arbuscular Mycorrhizal Fungi as Biocontrol Agents of Phytonematodes

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16.1 Introduction

The symbiotic associations between the plant roots and fungi are referred to as 'Mycorrhizae'. The term mycorrhiza from the Greek (*mykes* = mushroom or fungus and *rhiza* = root) can also be defined as 'a mutualistic symbiosis between plant and fungus, localized in a root or root-like structure in which energy moves primarily from plant to fungus and inorganic resources move from fungus to plant'. These symbiotic relationships are characterized by two-way movements of essential nutrients such as movement of carbon from plant to the fungus and movement of inorganic nutrients from fungus to plant, which are critical symbiotical linkages between the soil, root and plant. Mycorrhizal fungi in infertile soil help in uptake of nutrients, which results in improvement of plant growth. Mycorrhizal plants are always competitive and are able to withstand unfavourable environmental conditions compared to non-mycorrhizal plants. The vast majority of land plants form symbiotic associations with fungi and an estimated 95% of all plant species belong to diverse genera that characteristically form mycorrhizae. On the basis of morphology and anatomy, only seven types of mycorrhizae have come into general use so far. These are: ectomycorrhiza, endomycorrhiza or arbuscular mycorrhiza, ericoid mycorrhiza, arbutoid mycorrhiza, monotropoid mycorrhiza, ect-endomycorrhiza, and orchidaceous mycorrhiza.

Arbuscular mycorrhizal fungi (AMF), previously known as vesicular-arbuscular mycorrhizae (VAM), are mutualistic symbiotic associations between the roots of most vascular plants and a small group of fungi belonging to the new phylum Glomeromycota (Schussler *et al*., 2001). AMF are characterized by the presence of intercellular or intracellular hyphae, arbuscules which are branched hyphae involved in nutrient exchange (Fig. 16.1), extra-radicle mycelium that connect the root to the soil, and spores that are formed in the extra-radicle mycelium. Some fungal species also form intra-radicle structures referred to as vesicles (enlarged portions of hyphae that are filled with lipid bodies) (Fig. 16.2). Taxonomically, AMF belong to the phylum Glomeromycota, class Glomeromycetes, orders Archaeosporales, Paraglomerales, Diversisporales and Glomerales. Eight genera of AMF have been recognized, mainly on the basis of morphological characters of asexual spores (Schussler *et al*., 2001). These are *Glomus*, *Paraglomus*, *Sclerocystis*, *Scutellospora*, *Gigaspora*, *Acaulospora*, *Archaeospora* and *Entrophospora*, including approximately

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 Biocontrol Agents of Phytonematodes (eds T.H. Askary and P.R.P. Martinelli)

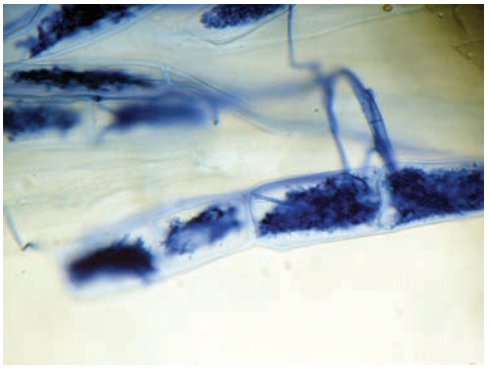

Fig. 16.1. Maize roots showing arbuscules of *Glomus mosseae.*

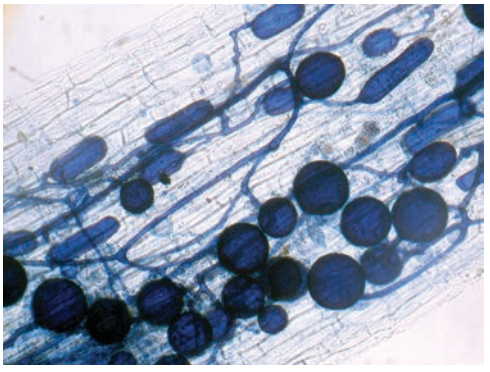

Fig. 16.2. Maize roots showing intraradicle hyphae and vesicles of *Glomus mosseae*.

214 species. Fossil and molecular evidence estimate that AM fungi date back to approximately 400 to 600 million years ago and played a critical role in the early establishment of land plants (Remy *et al*., 1994). They are believed to be asexual and colonization of roots can occur via spores, hyphae, or infected root fragments (Klironomos and Hart, 2002). Mycorrhizal root systems increase the absorptive area of roots by 10 to 1000 times, thereby greatly improving the ability of the plants to utilize the soil resource. Mycorrhizal fungi are able to absorb and transfer all the 15 major macro- and micronutrients necessary for plant growth. Mycorrhizal fungi release powerful chemicals into the soil that dissolve (otherwise unavailable). They interact with a wide range of organisms in the rhizosphere resulting in positive, neutral, or negative effect on the mycorrhizal plant association. Uptake of phosphorus in low fertile soils is a major role of these fungi and thereby protecting the plants from disease-causing organisms.

There are many instances where crop productivity is influenced by AMF symbioses, but there are still rather few examples where inoculation or management to increase AMF colonization is carried out as part of normal commercial practice. The effects of agricultural practices on AM fungi have recently been comprehensively reviewed (Jansa *et al*., 2006; Larsen *et al*., 2007). The factors rank (in decreasing order) as crop rotation, soil tillage, fungicide application and application of fertilizers in importance in modifying or reducing AM fungal populations. Other factors such as irrigation, burning and grazing, pollutants and topsoil removal are also relevant. The application of soluble phosphorus decreased root colonization (Abbott and Robson, 1984) with occasional reports of increase (Gryndler *et al*., 1990). Similarly, contradictory results have also been reported with nitrogen fertilizer (Baltruschat and Dehne, 1988; Gryndler *et al*., 1990; Liu *et al*., 2000). Biodynamic and organic farm management results in higher percentage colonization of roots of pasture and annual crops than conventional management (Ryan and Ash, 1999; Ryan *et al*., 2000). Therefore, use of AM fungi in the biological control for sustainable agriculture requires knowledge of culture systems that may affect their establishment and multiplication in the field.

16.2 Arbuscular Mycorrhizal Fungi and Nematode Interactions

Arbuscular mycorrhizal fungi are reported to occur profusely in agriculture crops. The plant-parasitic nematodes and AMF commonly occur together in the roots or rhizosphere of the same plant, each having a characteristic but opposite effect on plant growth. Among the various kinds of organisms engaged in biological control of nematodes, mycorrhizal fungi especially AMF fungi is now attracting greater attention as a potential biocontrol agent (BCA) (Harrier and Watson, 2004;

Akhtar and Siddiqui, 2008a; Sankaranarayanan and Sundarbabu, 2010; Hallman and Sikora, 2011; Vos *et al*., 2012a). Fox and Spasoff (1972) were the first to describe the interaction between these two groups of organisms. Baltruschat *et al*. (1973) were the first to show that plants pre-inoculated with AM fungus *Endogone mosseae* (=*Glomus mosseae*) were less susceptible to root-knot nematode infection. Since then numerous research papers have appeared from different parts of the world on the beneficial role of AMF against nematodes on a variety of crop plants as test host. AMF suppressed galling and reproduction of the root-knot nematodes on tomato (Kantharaju *et al*., 2010), banana (Jaizme-Vega *et al*., 1997), grape vine (Li *et al*., 2006) and black gram (Sankaranarayanan and Sundarbabu, 2009). The interactions of AMF with phytonematodes have been reviewed at regular intervals (Hussey and Roncadori, 1982; Smith, 1987; Ingham, 1988; Sikora, 1995; Pinochet *et al*., 1996; Borowicz, 2001; Habte and Schmitt, 2005; Hol and Cook 2005; Akhtar and Siddiqui, 2008a; Hallman and Sikora, 2011). As numerous reviews focus on AMF interaction effects, more emphasis has been laid in this chapter on the biological control potential of AMF against phytonematodes with special reference to root-knot nematode, *Meloidogyne* spp., cyst nematode, *Heterodera* and *Globodera* spp., lesion nematode, *Pratylenchus* spp. and burrowing nematode, *Radopholus similis*.

16.3 Biological Control of Root-knot Nematodes with Arbuscular Mycorrhizal Fungi

Biocontrol potential of AMF has been exploited mostly for the control of root-knot nematodes, *Meloidogyne* spp. in various crops. (Nikhita and Mahanta, 2013; Sankaranaraynan and Hari, 2013; Vos *et al*., 2013) (Table 16.1). Waceke *et al*. (2001) observed egg production of *Meloidogyne hapla* was reduced up to 75% and disease severity up to 71% in mycorrhizal plants. Sikora (1979) observed the reduced juvenile penetration of *Meloidogyne* spp. in tomato roots with AMF application. The other antagonistic effects of AMF against root-knot nematodes include reduced number and size of root-knot galls (Bagyaraj *et al*., 1979; Kellam and Schenck, 1980) and reduced nematode reproduction (Sikora, 1979). Sankaranarayanan (1995) studied the histopathological changes in black gram infested with *G. mosseae* and *Meloidogyne incognita*. The AMF penetrated the epidermis and invaded cortex giving rise to arbuscules and in some cortical cells proximity to nematode induced giant cells (Fig. 16.3).

Vos *et al*. (2013) studied the mechanism of AMF resistance against nematodes by using the suppression subtractive hybridization (SSH) technique to investigate plant genes that were specifically up-regulated in tomato roots (*Solanum lycopersicum* cv. Marmande) pre-colonized by the AMF *G. mosseae* (BEG 12). Mycorrhizal roots supported less nematode population than non-mycorrhizal roots, and they identified genes as defence, signal transduction, protein synthesis and modification. They found the involvement of the phenylpropanoid pathway and reactive oxygen species (ROS) metabolism for suppression of root-knot nematode infection in mycorrhizal tomato roots.

16.3.1 Integrated effect against root-knot nematodes

In general, a single BCA is used for biocontrol of plant disease against a single pathogen (Wilson and Backman, 1999). This may cause inconsistent results against all pathogens in soil environments. Dual inoculation with bioagents with a different mode of action will provide greater biocontrol against plant pathogens than individual inoculation (Sankaranarayanan and Sundarababu, 2001; Guetsky *et al*., 2002). Maximum growth of crop plants and control of root-knot nematodes, *Meloidogyne* spp. has been demonstrated by several workers by integration of AMF with fungal BCA *Paecilomyces lilacinus* (Bhat and Mahmood, 2000) and *Pochonia chlamydosporia* (Rao *et al*., 2003) than individual application of any BCA (Guetsky *et al*., 2002; Siddiqui and Akhtar, 2008, 2009). Different modes of action by these organisms probably resulted in synergistic effects in increasing

Table 16.1. Efficacy of arbuscular mycorrhizal fungi (AMF) against root-knot nematodes in some crops.

Crops	Root-knot nematodes	AMF	Effect on nematodes	References
Chilli	*Meloidogyne incognita*	*Glomus fasciculatum*, *G. mosseae*, *G. deserticola* and *Sclerocystis*	Suppressed nematode population and increased plant growth characters	Abhiniti *et al*. (2013)
Cucumber	*M. incognita*	*G. fasciculatum*	Significant suppression of galls, egg masses and nematode population in soil	Nikhita and Mahanta (2013)
Tomato	*M. incognita*	*G. mosseae* (BEG 12)	Mycorrhiza-induced resistance genes identified against root-knot nematodes	Vos *et al*. (2013)
Tomato	*M. javanica*	*G. mosseae*, *G. intraradices*	Mycorrhizal fungi improved plant growth characters and decreased nematode population	Sohrabi *et al*. (2012)
Solanum nigrum	*M. incognita*	*G. mosseae*	Improved plant growth characters in pre- and simultaneous application of AMF and suppressed nematode population	Robab *et al*. (2012)
Tomato	*M. incognita*	*G. mosseae*	Mycorrhiza-induced resistance against root-knot nematodes	Vos *et al*. (2012a)
Cucumber	*M. incognita*	*G. versiforme*	Suppressed nematode population	Chen *et al*. (2012)
Tomato	*M. incognita*	*G. mosseae*	Nematode penetration was reduced in mycorrhizal tomato roots	Vos *et al*. (2012b)
Tomato	*M. incognita*	*G. mosseae*	Suppressed root-knot nematodes throughout their entire early infection phase of root penetration and subsequent life stage development	Vos *et al*. (2012c)
Tomato and carrot	*Meloidogyne* spp.	Native AMF and commercial AMF	Field application of AMF suppressed nematode multiplication and root galling damage on both crops, indicating that the AMF persists and remains protective against root-knot nematodes over two crop cycles	Affokpon *et al*. (2011)
Tomato	*M. incognita*	*G. fasciculatum*	Suppressed nematode population and enhanced the growth and yield of tomato	Okon and Imuk (2011)
Tobacco	*M. incognita*	*G. intraradices*	Suppression of root-knot galls	Subhashini and Ramakrishnan (2011)
Cowpea	*M. incognita*	*G. mosseae*	AMF suppressed root-knot nematode reproduction on all varieties both in the screen house and field experiments	Odeyemi *et al*. (2010)
Black gram	*M. incognita*	*G. mosseae*	Application of AMF in different delivery methods suppressed nematode population by 14–49% under pot culture and 35–46% under micro-plot conditions	Sankaranarayanan and Sundarababu (2010)
Tomato	*M. incognita*	*G. fasciculatum* (SBI-G.f)	AMF increased plant growth and yield, besides reducing the root-knot population and root-knot index	Kantharaju *et al*. (2010)
Cucumber	*M. incognita*	*G. intraradices*	Enhanced plant growth and suppressed reproduction and/or galling of nematodes during the early stages of plant growth	Zhang *et al*. (2009)

Black gram	*M. incognita*	*G. mosseae*	Earlier application of AMF suppressed nematode population	Sankaranarayanan and Sundarababu (2009)
Lab lab	*M. incognita*	*Glomus* sp.	AMF improved plant growth and reduced root-knot nematode infestation	Ahmed *et al.* (2009)
Cucumber	*M. incognita*	*G. intraradices*, *G. mosseae*, *G. versiforme*	AMF suppressed nematode galling and egg mass production	Zhang *et al*. (2008)
Tomato	*M. incognita*	*G. fasciculatum*	Inoculation with AMF significantly reduced nematode population, number of galls and root-knot index	Shreenivasa *et al*. (2007a)
Tomato	*M. incognita*	*G. fasciculatum*	Inoculation with AMF significantly reduced the root penetration by nematode	Shreenivasa *et al*. (2007b)
Brinjal	*M. incognita*	*G. fasciculatum*	AMF significantly decreased the galls, egg masses and nematode population in soil and increased nitrogen, crude protein, phosphorus, potassium, total sugar, total phenol and total free amino acids in the roots	Borah and Phukan (2006)
Tomato	*M. incognita*	*G. fasciculatum*, *G. mosseae*	*G. fasciculatum* was more efficient than *G. mosseae* in controlling root-knot nematode infection	Verma and Nandal (2006)
Okra	*M. incognita*	*G. fasciculatum*	Suppressed nematode population	Verma *et al.* (2006)
Tomato	*M. incognita*	*G. fasciculatum*	AMF increased cost:benefit ratio and decreased nematode population	Kumar *et al*. (2006)
Grapevine	*M. incognita*	*G. versiforme*	AMF induced defence response against nematode in the mycorrhizal grapevine roots	Li *et al*. (2006)
Banana	*M. javanica*	*G. manihotis*	The mycorrhizal symbiosis reduced root galling and the population of nematodes in roots	Rodríguez Romero and Jaizme-Vega (2005)
Tomato	*M. incognita*	*G. fasciculatum*	AMF increased plant growth and yield, besides reducing the root-knot population and root-knot index	Kantharaju *et al*. (2005)
Black gram	*M. incognita*	*G. fasciculatum*	AMF decreased nematode penetration, delayed development, number of galls, egg masses, eggs per egg mass and final nematode population in soil	Bornali and Phukan (2004)
Tomato	*M. incognita*	*G. fasciculatum*	AMF suppressed nematode population	Pradhan *et al.* (2003)
Brinjal	*M. incognita*	*G. mosseae*	AMF suppressed gall index. Transplanting of mycorrhizal seedlings into root-knot nematode-infested soil performed better than non-mycorrhizal seedlings	Jothi and Sundarababu (2002)

Continued

Table 16.1. Continued.

Crops	Root-knot nematodes	AMF	Effect on nematodes	References
Black gram	*M. incognita*	*G. fasciculatum*	Seedlings inoculated with AMF before nematode infestation increased shoot length and reduced number of galls and egg masses	Mahanta and Phukan (2002)
Tomato	*M. incognita*	*G. mosseae*	AMF reduced gall index to 33% and final soil densities of *M. incognita* to 85%	Talavera *et al.* (2001)
Black gram	*M. incognita*	*G. fasciculatum*	Delivery of AMF inoculum by mixing with soil recorded the lowest gall index and nematode population	Sankaranarayanan and Sundarababu (2000)
Brinjal	*M. incognita*	*G. mosseae*, *G. fasciculatum*, *G. intraradices*, *G. fulvum*	The reproduction potential and the fecundity of *M. incognita* was significantly reduced due to AMF application	Jothi and Sundarababu (2000)
Menthol mint	*M. incognita*	*G. mosseae*	Maximum suppression of nematode reproduction was achieved with prior inoculation with VAM fungi	Ratti *et al*. (2000)
Black gram	*M. incognita*	*G. mosseae*	Suppressed nematode population	Sankaranarayanan and Sundarababu (1999)
Banana	*M. incognita*	*G. mosseae*	AMF favoured plant growth by enhancing plant nutrition and by suppressing nematode reproduction and galling during the early stages of micropropagated plant development	Jaizme-Vega *et al.* (1997)
Black gram	*M. incognita*	*G. fasciculatum*	Pre-application of AMF at 15 and 20 days earlier than nematode, suppressed the nematode population and increased biomass production	Sankaranarayanan and Sundarababu (1994)
Red and yellow pitaya	*M. incognita*	*G. manihotis*	AMF reduced the reproductive capacity of nematode	Palacino and Leguizamon (1991)

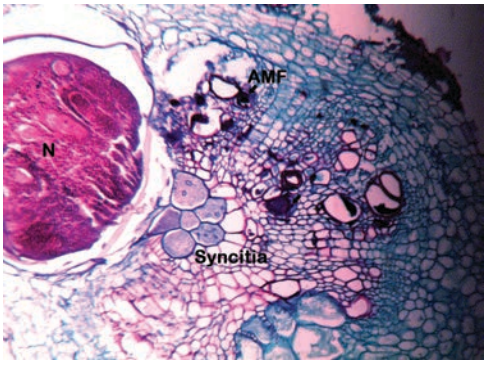

Fig. 16.3. Black gram roots showing root-knot nematode (N), giant cells and arbuscules of *Glomus mosseae.*

plant growth (Guetsky *et al*., 2002). Organic agricultural practices may favour increased number of AM spores in soil (Giovanetti and Avio, 1985). AMF sporulation is enhanced when soils are rich in organic amendments as reported by Johnson and McGraw (1988). Plant-parasitic nematodes can be controlled by application of organic soil amendments and this is considered an important component of an integrated nematode management programme. Many organic amendments, such as animal and green manures, compost and nematicidal plants are used for nematode control. Verma (2006) observed maximum reduction in nematode galling, egg mass formation and final soil population of *M. incognita* in okra when *Pseudomonas fluorescens*, *Glomus fasciculatum* and farmyard manure (FYM) were used combined. Neog and Islam (2008) integrated AMF *G. fasciculatum*, different organic amendments (neem cake, mustard oilcake, decaffeinated tea waste and sawdust) and carbofuran 3G individually and in combination against the root-knot nematode, *M. incognita*, infecting green gram. The dual application of all the organic amendments and carbofuran in combination with *G. fasciculatum* greatly decreased the number of galls, egg masses and final nematode population in the soil and increased the plant growth parameters as compared to individual application of these treatments. Compatibility of AMF with other BCA and organic amendments in controlling root-knot nematodes are presented in Table 16.2.

16.4 Biological Control of Migratory Endoparasitic Nematodes with Arbuscular Mycorrhizal Fungi

Migratory endoparasitic nematodes such as root lesion nematode, *Pratylenchus* spp. and burrowing nematode, *R. similis*, cause necrotic lesions in roots, which provide minimum space for AM fungi colonization. Adults and eggs of *Pratylenchus vulnus* were found in the same root tissue as AMF mycelium and vesicles (Pinochet *et al*., 1995a; Lopez *et al*., 1997). In perennial crops, nematode population build-up was sometimes decreased by mycorrhizal infection. This has been well documented for *Glomus intraradices* and *Radopholus citrophilus* on rough lemon seedlings (Smith and Kaplan, 1988) and apple and peach rootstocks, in which *G. mosseae* suppressed *P. vulnus* multiplication in the roots (Pinochet *et al*., 1995b). In these three cases, the number of nematodes per gram of root was significantly reduced in relation to low phosphorus non-mycorrhizal plants. However, in apple, in spite of the lower number of nematodes per gram of root in mycorrhizal rootstocks, the final nematode population was higher in these plants due to their larger root systems, capable of supporting a larger nematode population. Umesh *et al*. (1988) found that the number of *R. similis* in roots and soil was significantly lower when the AMF, *G. fasciculatum* was applied simultaneously or 7 days prior to nematode inoculation. Nyczepir and Halbrendt (1993) mentioned the beneficial role of AMF against migratory endoparasites, specifically root lesion nematodes *Pratylenchus*, which are largely involved in tree decline and orchard replant problems. It is evident from numerous research reports that AMF plays a beneficial role in suppressing lesion nematodes infesting different crops (Table 16.3). Root infection and multiplication of *Pratylenchus penetrans* were significantly reduced by pre-inoculation with AMF. AMF are crucial for the control of root-feeding nematodes in natural systems and locally operating mechanisms are involved in this process (de la Pena *et al*., 2006). Vos *et al*. (2012a) reported that *P. penetrans* is systemically reduced by *G. mosseae* in tomato.

Table 16.2. Combined efficacy of arbuscular mycorrhizal fungi (AMF), biocontrol agents and organic amendments against root-knot nematodes in some crops.

Crops	Root-knot nematodes	AMF	Biocontrol agents/organic amendments	Effect on nematodes	References
Tomato	*Meloidogyne incognita*	*Gigaspora gigantea*, *Glomus etunicatum*, *G. mosseae*, *G. deserticola*	*Paecilomyces lilacinus* (PL GoldTM)	Combined application suppressed root-knot galls	Udo *et al.* (2013)
Maize	*M. incognita*	*G. mosseae*	*Chromolaena odorata* powder	Suppressed nematode population	Odeyemi *et al.* (2013)
French bean	*M. incognita*	*G. fasciculatum*	*Trichoderma harzianum*	Significant decrease in nematode population	Gogoi and Mahanta (2013)
Field pea	*M. incognita*	*G. mosseae*	*Aspergillus awamori*, *Pseudomonas putida*, *P. alcaligenes*, *Paenibacillus polymyxa*	Combined treatment increased growth, enzymatic activities and reduced root galling and nematode multiplication	Akhtar and Panwar (2013)
Tomato and pepper	*M. incognita*	*G. intraradices*, *G. mosseae*	*Bacillus megaterium*	Synergistic affect led to reduction in nematode population	Flor-Peregrín *et al*. (2012)
Tomato	*M. incognita*	*G. intraradices*	Abamectin, fosthiazate	Abamectin alone and fosthiazate in the half dose mixed with AMF suppressed galls formation by 60.7% and 59.7%, respectively	Khalil (2012)
Black henbane (*Hyoscyamus niger*)	*M. incognita*	*G. intraradices*, *G. fasciculatum*, *G. mosseae*	*P. fluorescens*	Combined application of AMF and *P. fluorescens* reduced the population of nematode and increased the yield	Mishra *et al.* (2012)
Tomato	*Meloidogyne* spp.	*G. mosseae*	Organic fertilizers (manure simple, simple enriched manure ash)	Pre-inoculation with AMF reduced the galls and nematode population	Dodzi *et al*. (2012)
Tomato	*M. incognita*	*G. versiforme*, *G. mosseae*	*B. polymyxa*	AMF and PGPR together suppressed nematode population and improved plant growth than single inoculations	Liu *et al.* (2012)
Tomato	*M. incognita*	*G. intraradices*	*Heterorhabditis indica*	Application of EPN along with AMF reduced the galls, egg masses, number of eggs/egg mass and hatching rate of eggs	Hussaini and Kumar (2009)
Tomato	*M. incognita*	*G. intraradices*	*Steinernema abbasi*, *S. carpocapsae*, *S. feltiae*	Application of *S. carpocapsae* along with AMF reduced the galls, egg masses, number of eggs/egg mass and hatching rate of eggs	Hussaini *et al*. (2009)

Tomato	*M. incognita*	*G. aggregatum*	*B. coagulans*, vermicompost	Combined application of vermicompost, AMF and *B. coagulans* increased plant growth and number of galls and egg masses	Serfoji *et al*. (2010)
Cowpea	*M. incognita*	*G. geosporum*	*Rhizobium*	AMF + *Rhizobium* caused vigorous root growth and suppressed galling caused by nematode	Ugwuoke and Eze (2010)
Green gram	*M. incognita*	*G. fasciculatum*	Organic amendments (cake, mustard oilcake, decaffeinated tea waste and sawdust)	The dual application of AMF with organic amendments decreased the number of galls, egg masses and nematode population and increased the plant growth	Neog and Islam (2008)
Tomato	*M. incognita*	*G. intraradices*	*P. putida*, organic wastes (biosolids, horse manure, sawdust and neem leaf litter)	Combined use of neem leaf litter with *P. putida* plus AMF proved better in reducing galls and nematode multiplication	Siddiqui and Akhtar (2008)
Chickpea	*M. incognita*	*G. intraradices*	*P. straita*, *Rhizobium*	AM fungus with *P. straita* and *Rhizobium* caused increased in plant growth characters and suppressed nematode population	Akhtar and Siddiqui (2008b)
Chickpea	*M. incognita*	*G. intraradices*	*P. alcaligenes*, *B. pumilus*	Combined application of all the BCAs caused increase in plant growth characters	Akhtar and Siddiqui (2008c)
Tomato	*M. incognita*	*G. intraradices*	*Rhizobium etli*	Combined inoculation of AMF with *R. etli* led to additive effects in controlling nematodes than individual application of each treatment	Reimann *et al.* (2008)
Soybean	*M. incognita*	*G. mosseae*	*T. pseudokoningii*, *T. viride*, *P. lilacinus*, *A. niger*, *P. fluorescens*, *P. putida*	Suppression of nematode population and increase in plant growth characters	Oyekanmi *et al.* (2008)
Coleus	*M. incognita*	*G. mosseae*	*P. fluorescens*, *T. viride*, *P. lilacinus*, *Verticillium lecanii*	Suppression of nematode population	Seenivasan and Devrajan (2008)
Tomato	*M. incognita*	*G. intraradices*	*R. etli*	Combined inoculation of AMF and *R. etli* had additive effects in suppressing nematode population	Reimann *et al*. (2008)
Black gram	*M. incognita*	*G. fasciculatum*	*Rhizobium*	Combined application of AMF and *Rhizobium* reduced galls and egg masses	Bornali and Phukan (2007)
Black gram and green gram	*M. incognita*	*G. fasciculatum*	Neem cake	Reduction in nematode population	Borah *et al.* (2007)

Continued

Table 16.2. Continued.

Crops	Root-knot nematodes	AMF	Biocontrol agents/organic amendments	Effect on nematodes	References
Tomato	*Meloidogyne* spp.	*G. intraradices*	*Heterorhabditis* sp.	Compatible existence between the mycorrhiza and *Heterorhabditis* sp. in nematode suppression	Channashettar *et al.* (2007)
Okra	*M. incognita*	*G. fasciculatum*	*P. fluorescens*, FYM	Combined application of AMF with PGPR reduced nematode galling, egg mass formation and final soil population	Verma (2006)
Tomato	*M. incognita*	*G. intraradices*	*P. lilacinus*	Double treatment of mycorrhized seedlings with *P. lilacinus*, as seedling drench and pre-planting soil treatment resulted in reduced nematode damage to plants	Rumbos *et al*. (2006)
Brinjal	*M. incognita*	*G. fasciculatum*	Neem cake, carbofuran	Combined application significantly decreased root-knot index and nematode population in soil	Borah and Phukan (2004)
Brinjal	*M. incognita*	*G. fasciculatum*	*V. chlamydosporium*	Integration of AMF and *V. chlamydosporium* significantly increased plant growth and reduced root galling and nematode population	Rao *et al*. (2003)
Green gram	*M. incognita*	*G. fasciculatum*	Organic amendments (mustard cake, sawdust, poultry manure and decaffeinated tea waste)	Combination of AMF and decaffeinated tea waste reduced gall and nematode population	Neog and Gogoi (2003)
Tomato	*M. incognita*	*Glomus* sp.	*Pasteuria penetrans*	Combined application proved better in reducing nematode population	Talavera *et al*. (2002)
Black gram	*M. incognita*	*G. mosseae*	*Rhizobium*, phosphobacteria	Combined inoculation of *Rhizobium,* phosphobacteria and AMF reduced the gall index and nematode population	Sankaranarayanan and Sundarababu (2001)
Tomato	*M. incognita*	*G. mosseae*	*P. lilacinus*	Combined treatment suppressed nematode population	Bhat and Mahmood (2000)
Tomato	*M. incognita*	*G. mosseae*	*P. penetrans*	Combination of *P. penetrans* and AMF resulted in higher parasitism of female nematodes as compared to *P. penetrans* alone	Rao *et al.* (2000)
Tomato	*M. incognita*	*G. fasciculatum*	*T. harzianum*, neem cake	Suppressed nematode population	Reddy *et al*. (1998)
Brinjal	*M. incognita*	*G. mosseae*	*P. lilacinus*	Suppressed nematode population	Rao *et al.* (1998)

Tomato	*M. incognita*	*G. deserticola*	*P. penetrans*	Combined application significantly reduced the number of egg masses in root systems, and increased the parasitization of females by *P. penetrans*	Rao and Gowen (1998)
Tomato	*M. incognita*	*G. deserticola*	*V. chlamydosporium*	Integration of AMF with *V. chlamydosporium* resulted in a significant increase in the parasitization of nematode eggs by the fungus	Rao *et al*. (1997)
Black gram	*M. incognita*	*G. fasciculatum*	Leaf extracts of *Calotropis procera*, *Tagetes erecta*, *Catharanthus rosea*, *Bougainvillea spectabilis*	Addition of extracts recorded higher spore population and mycorrhizal colonization	Sankaranarayanan and Sundarababu (1996)
Tomato	*M. incognita*	*G. fasciculatum*	*C. procera*	Significant reduction of root-knot galls and number of eggs per egg mass	Rao *et al.* (1996)
Tomato	*M. javanica*	*G. mosseae*	*P. lilacinus*	Combined treatment completely inhibited root infection by *M. javanica*	Al-Raddad (1995)

Table 16.3. Efficacy of arbuscular mycorrhizal fungi (AMF) against migratory endoparasitic nematodes in some crops.

Crops	Migratory endoparasitic nematodes	AMF	Effect on nematodes	References
Tomato	*Pratylenchus penetrans*	*G. mosseae*	Systemic resistance developed in plants	Vos *et al*. (2012a)
Banana	*Radopholus similis*	*Glomus* sp.	Suppression of nematode population	Adriano-Anaya *et al.* (2011)
Banana	*R. similis*, *Helicotylenchus* spp.	*G. fistulosum*, *G. fasciculatum* and commercial inoculum	Suppression of nematode population	Ganan *et al.* (2011)
Maize	*P. zeae*	*G. mosseae*	Suppression of nematode population and increase in crop production	Oyekanmi *et al.* (2008)
Coffee	*P. coffeae*	*Glomus* sp.	Suppression of nematode population	Panneerselvam *et al*. (2008)
Dune grass *Ammophila arenaria*	*P. penetrans*	*Glomus* spp.	Root infection to plant by *P. penetrans* reduced	de la Pena *et al*. (2006)
Banana	*R. similis*	*G. fasciculatum*	AMF had adverse effect on nematode	Shreenivasa *et al.* (2006)
Wheat	*Pratylenchus* sp.	*G. mosseae*	AMF reduced root and the soil population of *Pratylenchus* sp. in rhizospheric regions	Anwar and Zaki (2005)
Crossandra	*P. delattrei*	*G. mosseae*	AMF suppressed the lesion nematode	Sundarababu *et al.* (2004)
Banana	*R. similis*, *P. coffeae*	*G. mosseae*	AMF suppressed nematode population build up in certain banana cultivars	Elsen *et al.* (2003a)
Banana	*R. similis*, *P. coffeae*	*G. mosseae*	Nematode population reduced in the presence of AMF	Elsen *et al*. (2003b)
Carrot	*P. coffeae*	*G. intraradices*	AMF provided the roots with increased protection against *P. coffeae* by suppressing nematode reproduction in the roots	Elsen *et al.* (2003c)
Carrot	*P. penetrans*	*G. mosseae*	Root infection by nematode reduced carrot growth, but soil inoculation with the spores of *Glomus* sp. compensated for the damage caused by nematode. Addition of *Glomus* spores to soil reduced *P. penetrans* soil densities by 49%	Talavera *et al.* (2001)
Apple	*P. penetrans*	*G. intraradices*, *G. mosseae*	Inoculation with VAM fungi reduced populations of nematode in roots and soil from the root zone. The effect of VAM fungi on nematode populations was most pronounced in fumigated soil	Forge *et al.* (2001a)
Apple	*P. penetrans*	*G. aggregatum*, *G. claru*, *G. etunicatum*, *G. intraradices*, *G. mosseae*, *G. versiforme*	Plants inoculated with *G. mosseae* supported fewer *P. penetrans* per gram of root than plants inoculated with other AM fungi	Forge *et al.* (2001b)

Coconut	*R. similis*	*A. bireticulata*, *Scutellospora coralloidea*, *G. macrocarpum*, *Sclerocystis rubiformis*	Suppression in nematode population	Sosamma *et al*. (1999)
Maize	*P. zeae*	*G. fasciculatum*	AMF, *Azospirillum* and *Phosphobacterium* when applied alone or in different combination reduced the population of nematodes	Sundarababu *et al.* (1998)
Plum	*P. vulnus*	*G. mosseae*, *G. intraradices*	Tolerance to plum rootstock against nematodes increased due to stimulation of plant nutrition and vegetative growth caused by AMF	Pinochet *et al.* (1998)
Coffee	*P. coffeae*	*Acaulospora mellea*, *G. clarum*	Early mycorrhizal inoculation (4 months before nematode inoculation) with either of the mycorrhizal species improved the tolerance to nematode in coffee plants	Vaast *et al.* (1998)
Coconut	*R. similis*	AMF mixture	AMF found effective in improving the plant growth and reducing the *R. similis* on coconut seedlings	Koshy *et al*. (1998)
Maize	*P. zeae*	*G. fasciculatum*	AMF suppressed nematode infection	Jothi and Sundarababu (1997)
Banana	*P. goodeyi*	*G. mosseae*, *G. aggregatum*	Early mycorrhizal inoculation appeared to increase host tolerance by enhancing plant nutrition and by reducing the nematode-induced lesions in the roots	Jaizme-Vega and Pinochet (1997)
Pear	*P. vulnus*	*G. intraradices*, *G. mosseae*	Early inoculation of AMF favoured pear rootstock growth and conferred protection against nematode by inhibiting nematode reproduction and by enhancing plant nutrition	Lopez *et al.* (1997)
Ragi	*P. zeae*	*G. fasciculatum*	*G. fasciculatum* individually and in combination with biofertilizers resulted in higher yields per plot of ragi by reducing the multiplication rate of the nematodes	Sundarababu *et al.* (1996)
Cherry	*P. vulnus*	*G. intraradices*	Early mycorrhizal infection of cherry rootstock increased plant growth in the presence of nematodes	Pinochet *et al*. (1995a)
Peach	*P. vulnus*	*G. mosseae*	Suppression of nematode population	Pinochet *et al*. (1995b)
Quince	*P. vulnus*	*G. intraradices*	Inoculation with AMF favoured quince growth and conferred protection against *P. vulnus* by improving plant nutrition	Calvet *et al*. (1995)
Pineapple	*P. brachyurus*	*Glomus* sp.	Early application of AMF decreased nematode number per gram of root	Guillemin *et al.* (1994)
Apple	*P. vulnus*	*G. mosseae*	AMF suppressed nematode infestation level per gram of root	Pinochet *et al*. (1993)

16.5 Biological Control of Cyst Nematodes with Arbuscular Mycorrhizal Fungi

The beneficial role of AMF for cyst nematode control has been evidenced by several reports (Francl and Dropkin, 1985; Jain and Sethi, 1987, 1988). Li *et al.* (2002) conducted experiments on interactions between *Gigaspora margarita*, *G. fasciculatum*, *G. intraradices*, *G. mosseae*, *Glomus versiforme* and *Heterodera glycines* race 4 on soybean cv. Kaiyu 10, which is susceptible to soybean cyst nematode (SCN). It was observed that AM fungi could significantly decrease infection on SCN, reduced disease severity, the number of cysts on roots, the number of cysts and the second-stage juveniles (J_2) in the rhizospheric soil, and the number of eggs per cyst. Combined inoculation of *G. mosseae*, *Trichoderma harzianum* and *Verticillium chlamydosporium* reduced nematode multiplication and wilting index of pigeonpea caused by *Heterodera cajani* and *Fusarium udum* (Siddiqui and Mahmood, 1996). The biocontrol of cyst nematodes, *Heterodera* and *Globodera* spp. by AMF are presented in Table 16.4.

16.6 Biological Control of Other Nematodes with Arbuscular Mycorrhizal Fungi

An inconsistent interaction is often found between ectoparasitic nematodes and AM fungi. The ectoparasitic nematode, *Xiphinema index* commonly known as dagger nematode, which is a vector of grapevine fanleaf virus (GFLV), provokes gall formation and has the ability to cause severe damage to the root system of grapevines. Mycorrhiza formation by *Glomus* (syn. *Rhizophagus*) *intraradices* BEG141 reduced both gall formation on roots of the grapevine rootstock SO4 and population of nematode in the rhizosphere. Suppressive effects increased with time, however simultaneous inoculation of nematode and AMF resulted in lesser suppression as compared to post-inoculation of nematode. When the split-root system was used, it decreased the development of *X. index* as shown in mycorrhizal and non-mycorrhizal parts of mycorrhizal root systems. This indicates that both local and systemic induced bioprotection mechanisms were active against the ectoparasitic nematode. The data showed priming of grapevine defence responses by the AM fungus and transmission of a plant-mediated signal to non-mycorrhizal tissues. The responses of grapevine gene during AM-induced local and systemic bioprotection against *X. index* point to biological processes related either as direct effects on the nematode or protection against nematode-imposed stress for maintaining root tissue integrity (Zhi-Peng *et al*., 2012). Sorghum roots infected with AM fungi had lower numbers of *Tylenchorhynchus vulgaris* and *Helicotylenchus dihystera* (Jain and Hasan, 1986). Lucerne pre-inoculated with *G. fasciculatum* significantly reduced the adverse effects of *T. vulgaris* on plant biomass (Jain *et al*., 1998). The efficacy of commercial formulations of AMF, plant growth-promoting rhizobacterium (PGPR) and the antagonistic fungus against reniform nematode, *R. reniformis* in cotton was studied by Sreenivasan and Sundarababu (2007), who found that *G. mosseae*, *P. fluorescens* and *Trichoderma viride* as soil application improved the plant growth and yield of cotton. Soil application of *G. mosseae* recorded maximum reduction of nematode population in roots (72.1%) and soil (81.1%).

16.7 Mode of Action of Arbuscular Mycorrhizal Fungi Against Phytonematodes

There are many mechanisms involved during interaction of AMF and plant-parasitic nematodes on a host. Whipps (2004) indicated the following possible modes of action of AMF against plant pathogens: (i) direct competition; (ii) enhanced plant growth; (iii) biochemical changes and induced resistance; and (iv) development of an antagonistic microbiota.

Vos *et al*. (2012b) reported that root exudates of *G. mosseae* on tomato roots affected motility of *M. incognita*. Li *et al*. (2003) suggested that *G. fasciculatum* could first activate the defence mechanism of soybean, and then make the roots of soybean react quickly to

Table 16.4. Efficacy of arbuscular mycorrhizal fungi (AMF) against cyst nematodes in some crops.

Crops	Cyst nematodes	AMF	Effect on nematodes	References
Soybean	*Heterodera glycines* race 4	*G. fasciculatum*	AMF activate the defence mechanism of soybean by activating phenyl-alanine ammonia lyase (PAL) and chitinase, which play a critical role in the resistance induced by AM fungi against the nematode	Li *et al*. (2003)
Soybean	*H. glycines* race 4	*Gigaspora margarita*, *G. fasciculatum*, *G. intraradices*, *G. mosseae*, *G. versiforme*	AMF significantly decreased nematode damage, reduced disease severity, the number of cysts on roots, the number of cysts and the second-stage juveniles (J_2) in the rhizospheric soil and the number of eggs per cyst	Li *et al*. (2002)
Cowpea	*H. cajani*	*G. fasciculatum*	AMF suppressed cyst nematode	Nageswari and Sundarababu (1998a)
Cowpea	*H. cajani*	*G. fasciculatum*	AMF hampered the root invasion by *H. cajani* leading to reduction in cyst population	Nageswari and Sundarababu (1998b)
Pigeonpea	*H. cajani*	*G. mosseae*	Combination of AMF, *P. lilacinus* and *P. fluorescens* have reduced nematode multiplication and wilting	Siddiqui *et al.* (1998)
Pigeonpea	*H. cajani*	*G. mosseae*	AMF combined with *T. harzianum* and *V. chlamydosporium* reduced nematode multiplication and increased plant growth	Siddiqui and Mahmood (1996)
Pigeonpea	*H. cajani*	*G. fasciculatum*	Combined treatment of AMF, *Bacillus subtilis*, *Bradyrhizobium japonicum* increased plant growth, nodulation, phosphorus contents and reduced nematode multiplication	Siddiqui and Mahmood (1995)
Soybean	*H. glycines*	*G. intraradices*	AMF plants had greater nematode tolerance	Price *et al*. (1995)
Soybean	*H. glycines*	*Glomus* spp.	The effect of AMF on cyst nematode varied with time. Number of nematodes in roots and soil were decreased up to 73% by AMF	Tylka *et al*. (1991)
Cowpea	*H. cajani*	*G. fasciculatum*, *G. versiforme*	Prior application of AMF suppressed nematode cyst development	Jain and Sethi (1988)
Cowpea	*H. cajani*	*G. fasciculatum*, *G. versiforme*	AMF had adverse effect on cyst production	Jain and Sethi (1987)
Potato	*Globodera pallida*, *G. rostochiensis*	*Glomus* spp. (commercial inocula)	AMF had adverse effect on *G. pallida*	Deliopoulos *et al.* (2011)
Potato	*G. pallida*	*Glomus* spp. (commercial inocula)	AMF enhanced the efficacy of carbamate nematicides against *G. pallida*	Deliopoulos *et al.* (2010)
Potato	*G. pallida*	*Glomus* spp. (commercial inocula)	AMF reduced *G. pallida* multiplication by dual mechanism involving stimulation of nematode hatching and inhibition of root invasion	Deliopoulos *et al*. (2007)
Potato	*G. pallida*, *G. rostochiensis*	*Glomus* spp. (commercial inocula)	AMF used as a component in integrated nematode management suppressed the population of *G. pallida*	Ryan *et al.* (2003)

H. glycines infection. PAL and chitinase play a critical role in the resistance induced by *G. fasciculatum* against the nematode. β-1,3-glucanase activities in roots of plants pre-inoculated with *G. fasciculatum* and post-inoculated with SCN were lower than those in roots treated with pre-inoculated *G. fasciculatum*. However, the role of β-1,3-glucanase in protecting soybean from infection of nematodes was not obvious.

The systemic induction of defence in mycorrhizal root systems has been reported against *X. index* in grapevine (Hao *et al*., 2012), *Pratylenchus coffeae* and *R. similis* in banana (Elsen *et al*., 2008) and *M. incognita* in tomato (Vos *et al*., 2012a). Little is known about the molecular basis of mycorrhiza-induced resistance against nematodes in roots. Activation of a class III chitinase gene was reported in mycorrhizal grapevine roots after root-knot nematode infection (Li *et al*., 2006). Hao *et al*. (2012) reported that genes identified for nematode control relate either to direct effects against the nematode or to the maintenance of root tissue integrity while the plant is under nematode attack.

Alguacil *et al*. (2011) by applying molecular methods studied whether galls produced by *M. incognita* infection in *Prunus persica* roots are colonized by AMF, and determined the changes in AMF composition and biodiversity mediated by infection with the root-knot nematode. It was observed that galls produced in *P. persica* roots due to infection with *M. incognita* were colonized extensively by a community of AMF, belonging to the families Paraglomeraceae and Glomeraceae, that was different from the community detected in roots. Although the function of the AMF in the galls is still unknown, it is hypothesized that they act as protection agents against opportunistic pathogens.

ZhiPeng *et al*. (2012) observed local and systemic mycorrhiza (*G. intraradices*) induced protection against *X. index* that involves priming of defence gene responses in grapevine. Vos *et al*. (2012a) attempted to find the mode of action of AMF against *M. incognita* and *P. penetrans* in tomato. It was found that *M. incognita* and *P. penetrans* are systemically reduced by the AMF, *G. mosseae*. Vos *et al*. (2013) found that up-regulated gene expression in the biocontrol interaction between mycorrhizal tomato and *M. incognita* existed and new insights were found into the molecular mechanisms underlying the mycorrhiza-induced resistance against root-knot nematodes.

Plants can develop a defence reaction to AMF infection and this 'mycorrhiza-induced resistance' (MIR) provides systemic protection against a wide range of pathogens. This is similar to systemic acquired resistance (SAR) after pathogen infection and induced systemic resistance (ISR) following root colonization by non-pathogenic rhizobacteria. MIR is a cumulative effect of plant responses directly to mycorrhizal colonization and indirect immune responses to ISR-eliciting rhizobacteria in the mycorrhizosphere (Cameron *et al*., 2013).

16.8 Conclusions and Future Area of Research

The exploitation of AMF in the biocontrol of phytonematodes on various crops is an environmentally feasible method. With the concept of health hazards due to nematicides, there is a growing interest in AMF in the field of biological control. The last three decades have seen more research and a greater number of papers have appeared on AMF in laboratory and in field studies. AMF associations have been shown to reduce damage caused by soil-borne plant pathogens and certain species and strains of AMF have biocontrol potential towards sedentary and migratory endoparasitic nematodes. Focus should be given to prior establishment of AMF in the field by planting white clover and related plants to increase the mycorrhizal inoculum in the soil, thus allowing rapid and high levels of root colonization of the following main crop. Suitable combinations of AMF, *Rhizobium*, PGPR, *Pseudomonas*, *Bacillus*, nematophagous fungi such as *P. lilacinus*, *P. chlamydosporia* and *Arthrobotrys* spp. may increase plant growth and control plant nematodes. The obligate nature of AMF is a major bottleneck in its mass production and emphasis has been given on large-scale production of AMF with root organ culture methods

(Fortin *et al*., 2002). Molecular-level mechanisms are needed in the symbiotic association between plants and AMF in controlling phytonematodes, which may lead to newer biocontrol strategies for nematode management in the future.

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Part VII

Predatory Nematodes

17 Predatory Nematodes as Biocontrol Agents of Phytonematodes

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17.1 Introduction

Nematodes are multicellular triploblastic invertebrates with a pseudocoel that belong to the phylum Nematoda of the kingdom Animalia. They are ubiquitous in nature, inhabiting a very broad range of environments largely as marine or terrestrial inhabitants. Among their terrestrial forms, soil nematodes are very small (generally 0.3–0.5 mm long as adults) wormlike animals with a high diversity (commonly >30 taxa) predominating over all other soil animals in species as well as in number (commonly millions per square metre; Yeates, 1979; Bernard, 1992).

Soil nematodes feed on soil organisms belonging to a broad range of groups. Based on feeding behaviour, they can be classified into different trophic groups, such as bacterial feeders, fungal feeders, algal feeders, animal predators, omnivores and plant parasites (Freckman and Caswell, 1985; Yeates and Bongers, 1999). All of these nematodes within the various trophic groups are in intimate contact with their surroundings by living in the continuity of soil water films, where most soil microorganisms interact with biotic and abiotic environmental factors; they occupy several positions in the soil food-web and are very influential, positively or negatively, on the possible interactions among components of the agroecosystem–soil–food web (Freckman and Caswell, 1985; Ferris *et al.*, 2001). In the soil ecosystem, all nematode trophic groups are associated with soil health and functioning, playing important roles in the mineralization of nitrogen for plant growth and distribution of biomass through grazing on microbial decomposers, immobilizing nitrogen in the live biomass and secreting ammonium, sometimes damaging plants in agroecosystms (Freckman and Caswell, 1985; Ingham *et al.*, 1985; Beare, 1997; Ferris *et al.*, 1998; Neher, 2001).

Plant-feeding nematodes with armament of a hollow stylet cause diverse types of damage to plants, including the induction of diseases, incitation and aggravation of non-nematode diseases due to other microbial plant pathogens, such as fungi and bacteria, and the transmission of other organisms pathogenic to plants, most notably by plant virus-transmitting nematodes such as *Xiphinema*, *Longidorus* and *Trichodorus* (Pitcher, 1982; Dowler and Van Gundy, 1984). All damage caused to crop plants by plant-parasitic nematodes worldwide has been estimated to cost over US$100 billion annually (Oka *et al.*, 2000; Chitwood, 2003).

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Several potential control methods exist: the use of chemicals (nematicides), cultural practices such as rotation, the use of nematode-resistant plant cultivars, quarantine and possibly biological control, all of which can contribute to nematode control in integrated pest management (IPM) (Thomason and Caswell, 1987). All of these control methods have advantages and drawbacks in relation to their practical applications, such as control efficacy, economic reliability and ecological safety. Use of chemical nematicides, which contrasts greatly with biological control methods, is one of the primary means of combating plant-parasitic nematodes and has proven to be the most effective means of control in modern crop production, especially in high-value horticultural crops in which these methods are economically practical (Hague and Gowen, 1987). However, nematicides (especially fumigant types) are too expensive to be applied to many field crops and often do not provide long-term suppression of nematodes. Nematicides are also very toxic and cause potential negative impacts on the environment and human health, which has led to their total ban or restricted use in recent years (Khan and Kim, 2005). Alternatively, environmentally friendly management tactics for plant-parasitic nematodes such as the use of biological control agents (natural enemies) are required as a substitute for chemical control tactics (Noling and Becker, 1994).

The biological control of plant-parasitic nematodes is generally regarded as the suppression of nematode populations by living organisms and their by-products, including higher plants, fungi, bacteria and animals antagonistic to the nematodes (Brown, 1982). Among these living organisms excluding higher plants, nematode-trapping and endoparasitic fungi (including pseudo-fungi) are the most successful, followed by predatory nematodes, contributing 73–76% and 7–13%, respectively, of the total research efforts based on the number of references available between 1987 and 2002 (Kerry, 1987; Bilgrami, 2008). A few microbial products are currently being sold for the control of plant-parasitic nematodes, while over 80 products with biological control activity against plant pathogens are potentially available in the market (Whipps and Davies, 2000). Considering the research efforts made on biological control agents during the past years, products for predatory nematodes that are practical and reliably effective in the control of plant-parasitic nematodes are seldom available in the commercial market.

Decades have passed since the potential for predatory nematodes to control populations of plant-parasitic nematodes was first proposed by Cobb (1917). Profound research efforts have focused on the potential of predatory nematodes in nematode pest control. Two reviews by Khan and Kim (2007) and Bilgrami (2008) addressed and fully discussed the role and biological control potential of predatory nematodes in dealing with plant-parasite nematodes. Here, the attributes of predatory nematodes required for the control of pest nematodes are addressed and compared among predatory nematodes with different feeding types, illustrating both theoretical principles and practical problems to gain new insights in the development of effective biological control agents of plant-parasitic nematodes, largely referring to the two review papers mentioned above.

17.2 Attributes of Biocontrol Potential

Biological control, a method of controlling pests using other living organisms referred as biological control agents (BCAs), relies on their antagonism such as competition, antibiosis, parasitism and predation to protect plants through the exclusion, displacement or inhibition of pests (Cook and Baker, 1983). For the practical use of BCAs, their biological attributes are required in relation to their efficacy and usability consisting of stability, safety and ease of application (Fravel, 2005; Droby *et al.*, 2009). This can be applied to predatory nematodes, for which biological attributes for the control of plant-parasitic nematodes include the following: predation ability and prey searching capability (colonizing ability, persistence or dispersal, prey preference and prey spectrum) for predator's control efficacy; and mass production, longevity and stability, and

ease of application for predators' usability (Kerry, 1987; Bilgrami, 2008). In the usability of BCAs, the safety of predatory nematodes is very important for their general use but is not discussed here because none of the predatory nematodes has been reported to provide critically adverse influence on humans, animals or beneficial soil biota. Rather, predatory nematodes promote soil health by regulating the composition of nematode communities at all trophic levels (consequently controlling the microbial communities) through their prey density-dependent predatory activities, maintaining soil biodiversity and stabilizing two critical ecological processes, i.e. nitrogen cycling and decomposition (Freckman and Casswell, 1985; Neher, 2001; Mulder *et al.*, 2011).

17.2.1 Predation ability

Predation ability is an important attribute to be examined in selecting an efficient predatory nematode for controlling plant-parasitic nematodes. Prey predation is composed of prey capturing and feeding, which is divided into different predation phases: i.e. the encounter with prey, attack response and subsequent feeding (composed of attack, extracorporeal digestion and ingestion) (Bilgrami and Jairajpuri, 1989b; Bilgrami, 2008). All predation phases are related to the ability of prey capturing, feeding, or both, contributing to the lethality of the prey for reducing prey populations; their roles in the biological control of plant-parasitic nematodes are described as follows (Fig. 17.1).

Encountering of prey

The encountering of prey is related to the capturing ability of predatory nematodes and is determined by the predator–prey contacts, which are established either by accidental contacts during the random movement of predatory nematodes such as mononchid predators (Grootaert and Maertens, 1976) or by intentional contacts of predatory nematodes moving toward the prey nematodes in chemosensory response to prey secretions (kairomones or attractants) in cutting and sucking-type nematodes (diplogasterid predators such as *Mononchoides* and *Butlerius*) and stylet-bearing (or puncturing and sucking-type) nematodes such as dorylaimid predators (*Mesodorylaimus* and *Aquatides*) (Bilgrami and Jairajpuri, 1988a; Bilgrami, 1997; Bilgrami and Pervez, 2000; Bilgrami *et al.*, 2000, 2001; Pervez and Bilgrami, 2000). The intentional contact of predators with prey secretions may not only increase the probability of the establishment of predator–prey contacts but also derive them aggregating around the feeding sites where the prey density is high. In these respects, predatory nematodes having intentional movements possess higher capturing ability than those with accidental contacts, thus providing the former with higher predatory ability than the latter. The responses of predatory nematodes are differentiated based on various attributes of the predators such as their innate behaviour, their preference for particular prey species, the chemical composition, concentration, quality and quantity of prey attractants, the formation of a minimum perceptible attraction gradient for prey attractants and the minimum response threshold of the predators, all of which should be influenced by prey density, period of prey incubation and predator starvation, temperature, and distance of predators from prey (Pervez and Bilgrami, 2000). These aspects indicate that predatory nematodes capable of responding to kairomones emitted from prey may have a high probability of not only encountering prey, but also a certain

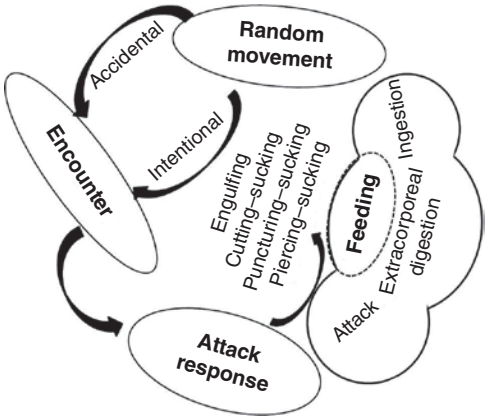

Fig. 17.1. Predation phases in predatory nematodes.

degree of prey-specificity, which is a desirable characteristic in the biological control of plant-parasitic nematodes.

Attack response

Attack response is derived from head probing, feeding apparatus movements and oesophageal pulsations after predator–prey contacts to secure successful attack by the predators. The vigour and extent of attack response and prey-probing vary depending on the predator, from aggressive (vigorous and broad) responses in mononchids (*Prionchulus* and *Mylonchulus*) to vigorous and confined or gradual and restricted responses in dorylaimids (*Labronema* and *Dorylaimus*), rapid side-to-side probing (lip rubbing) in mononchids (*Mononchus*) and vigorous or just head shaking and lip rubbing against the prey nematode body in diplogasterids (*Mononchoides* and *Butlerius*). The significance of these variable attack responses in relation to the abilities of prey feeding (biological control potentials) has been poorly documented; however, these may be determined in relation to the attack by predatory nematodes, the success of which is governed by the attack responses to prey nematodes (Bilgrami, 2008).

Feeding apparatus and feeding mechanisms

Four types of feeding apparatus and feeding (ingestion) mechanisms exist (Fig. 17.2): (i) the engulfing type in mononchid predators that bite (hold) the prey with teeth and a strongly sclerotized buccal cavity (totally called buccal armature); using high oesophageal suction, they engulf and swallow the prey whole or shred the prey body before feeding with the support of oesophageal muscle contractions to pull the prey into the buccal cavity; (ii) the cutting–sucking type in diplogasterid predators possessing a comparatively smaller buccal cavity but armed with teeth for cutting the prey body and then sucking the body contents using oesophageal suction; (iii) the puncturing–sucking type in dorylaimid predators possessing a feeding apparatus of hollow odontostyle or non-hollow mural tooth or onchia, which is used to puncture the prey body and suck the body contents with the help of oesophageal suctions; and (iv) the piercing–sucking type in aphelenchid predators such as *Seinura* possessing a slender stylet with a fine lumen, with which they penetrate prey cuticle and inject oesophageal secretion into the prey body to paralyse the prey and digest prey body contents before ingestion with the help of oesophageal suctions (Hechler, 1963; Yeates, 1969; Groottaert *et al.*, 1977; Wyss and Grootaert, 1977; Shafqat *et al.*, 1987; Bilgrami and Jairajpuri, 1989b; Khan *et al.*, 1991).

Feeding is composed of attack, extracorporeal digestion and ingestion. Feeding is initiated by the attack of predatory nematodes to cut and penetrate the cuticle of prey nematodes using side-to-side rib rubbing with simultaneous movements of the feeding apparatus and is completed with the ingestion of the prey body contents using the feeding apparatus in combination with oesophageal suctions (Bilgrami and Jairajpuri, 1989b). Digestion of the prey body contents to small food particles (termed as extracorporeal digestion) is necessary for stylet-bearing predatory nematodes such as dorylaimids and aphelenchids to ingest them through a narrow stylet lumen in transit to the intestine (Bilgrami and Jairajpuri, 1989b). Also, extracorporeal digestion is required in diplogasterid predators with a small oral aperture; the complex food globules should be digested or at least broken into small particles before they are ingested through the small oral aperture. Mononchid predators, however, have a wide oral aperture through which the food materials are ingested prior to digestion. The presence or absence of preliminary digestion of the prey body contents may be just a matter of feeding processes most fitted to the different feeding habits with little relations to the predatory ability of the predators.

Biological control efficiencies of predatory nematodes in relation to their predation ability can be evaluated by prey mortality and feeding time: prey mortality is related to the strike rate (the number of predatory nematodes having attacked the encountered prey nematodes relative to the total number of predatory nematodes encountered with the prey nematodes) and prey susceptibility (the number of prey nematodes with wounds

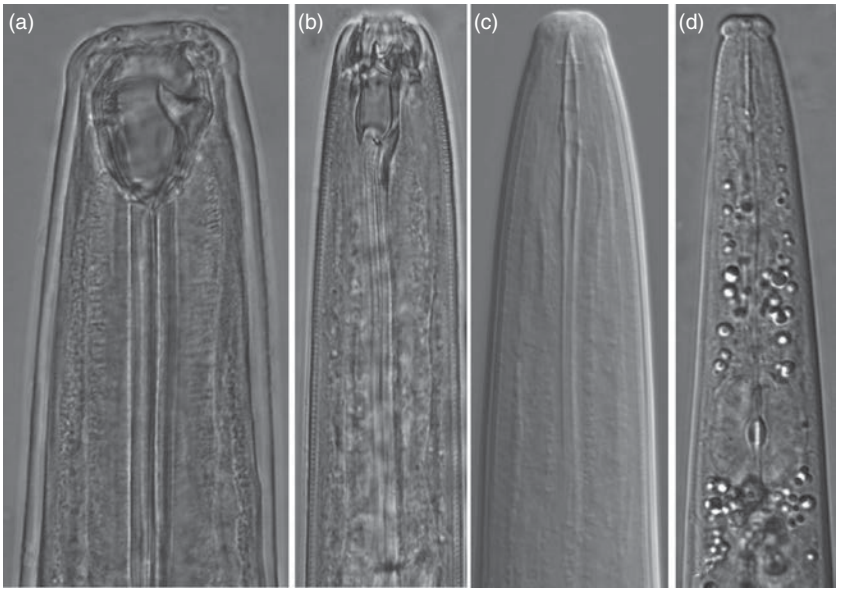

Fig. 17.2. Anterior region of nematodes showing the types of feeding armament: (a) engulfing type found in mononchids; (b) cutting–sucking type in diplogasterids; (c) puncturing–sucking type in dorylaimids; and (d) piercing–sucking type in aphelenchids.

relative to the total number of prey nematodes attacked by the predators) (Bilgrami, 2008). The comparison of predation ability among mononchid predator (*Mononchus aquaticus*) and dorylaimid predators (*Dorylaimus stagnalis* and nyglolaimid *Aquatides thornei*) shows that all predators examined have the highest strike rates with the highest prey susceptibility to predation against endoparasitic nematodes, and next against saprophagous nematodes, among which *M. aquaticus* has the higher strike rate compared to the other two against all trophic groups of prey nematodes (Bilgrami, 1992). Feeding time for consuming a single endoparasitic nematode prey is longer in the dorylaimids than in *M. aquaticus*; the mononchids are capable of attacking large ectoparasitic nematodes such as *Hoplolaimus*, *Scutellonema* and *Hemicycliophora* (*Hemiconemoides*), which are not stricken by the two dorylaimids, diplogasterids (*Mononchoides longicaudatus* and *Mononchoides fortidens*) and aphlenchids (*Seinura paratenuicaudata*) (Bilgrami and Jairajpuri, 1989a; Bilgrami, 1995; Vats *et al.*, 2004). The degree of prey mortality would be the product of the predator's strike rate and prey susceptibility as the real number of dead prey nematodes is derived from the number of susceptible nematodes attacked (stricken) by the predators. In this respect, mononchids can be used as reliable BCAs owing to their high strike rates and the high sensitivity of their prey. However, their predation abilities are diminished significantly by their low prey capturing abilities due to accidental predator–prey contacts so that overall predation ability is relatively lower than that of the other predatory nematodes.

17.2.2 Prey searching capability

Natural enemies must be present for a certain period of time for the biological control of plant-parasitic nematodes to be realized, which requires the predatory nematodes to have high degrees of prey searching capacity owing first to their high colonizing ability and durable distribution in soil habitats (persistence and dispersal). The prey searching abilities of predatory nematodes differ depending on predators (and possibly against prey by prey) and are influenced by biotic and abiotic soil

factors. Nematodes that increase rapidly in number under favourable conditions can be considered to have high colonizing ability (termed as colonizers comparable to *r*-strategists) due to their short life-cycles, high fecundity and tolerance to disturbance and eutrophication (Bongers, 1990). Diplogasterid predators are the first colonizers, followed by aphlenchid predators (*Seinura* spp.) and then dorylaimid predators, because of their high prey searching capability due to intentional contacts with prey. Dorylaimids (especially Nygolaimidae), however, are the first persisters (*K*-strategists), followed by mononchids with low colonizing abilities and living in habitats with a long durational stability without disturbances from farming practices (Bongers and Bongers, 1998; Yeates and Bongers, 1999). On an individual nematode basis, mononchids and dorylaimids are assumed to have higher persistence because of their long lifespans when compared with diplogasterids and aphelenchids; however, they differ in their persistence at the population level because of the difference in their distribution under natural conditions. Mononchid predators distribute in confined habitats, diminishing their persistence, which may decrease their potential for the biological control of plant-parasitic nematodes, while dorylaimid predators distribute widely in various soil habitats, compensating for their low colonizing ability and increasing their persistence in soil habitats.

Despite the colonizing ability and persistence (dispersal), practical approaches for the biological control of plant-parasitic nematodes with predatory nematodes can enhance their biological control efficiencies, which can be differentiated into introduction, inundation and natural control in combination with their persistence and/or dispersal (Kerry, 1987). For predatory nematodes with high colonizing abilities and a relatively high prey searching capability, such as diplogasterids, aphelenchids and possibly dorylaimids, introduction can be applied in soils in which the predatory nematode is normally absent so that it can be spread and establish itself in soils to provide durable pest control. For those with low colonizing abilities such as mononchids and dorylaimids (with low prey searching capability), inundation or natural control can be used as a practical approach depending on their persistence or dispersal. Inundation of those with low persistence and low dispersal (mononchids), whereby the predators are introduced in large numbers into confined areas to obtain rapid pest control with frequent applications due to their low persistence and distribution in soil, and natural control for those with a low colonizing ability but a large distribution (comparable to high persistence in terms of an increased probability of predator–prey contacts; dorylaimids) should be applied to foster the conditions favoured by the predatory nematodes so as to sustain their effective population densities (Kerry, 1987).

Prey preference (in terms of plant-parasitic nematodes) is a key feature of predatory nematodes, governing the efficient biological control of plant-parasitic nematodes in the sense that the predators with a narrow and discriminative prey range can encounter and attack target prey with little loss of time and energy used for searching non-target prey, so that their searching ability for target pests is improved. In contrast, predators with high prey specificity have limitations in their use for biological control candidates because of their decreased ability for attacking a diverse spectrum of plant-parasitic nematodes and due to the difficulties in their mass culturing (Bilgrami *et al.*, 2005; Bilgrami, 2008). Also, natural enemies with high host specificity tend to have limitations in terms of survival and duration in the field with concomitant depletion of their target organisms (Deacon, 2006).

Bilgrami *et al.* (1986) examined the prey preference of mononchid predators by analysing predator intestinal contents and showed that the predator specimens containing saprophytic nematodes were more numerous than those containing other prey nematodes, probably due to the relative abundance of saprophytic nematodes in soil habitats, which needs to be examined in detail. In another study, the examination of predator–prey systems for mononchid predators showed that predatory adults feed on prey nematodes in relation to prey body size (with bodies five times larger than their prey) without discriminating between the taxonomic groups or trophic types of prey nematodes (Mulder *et al.*, 2011), which

implies that mononchid predators with wide oral apertures (engulfing type) are supposed to be more dependent upon the prey body size because they ingest the whole or part of their prey without extracorporeal digestion. Diplogasterids can feed on prey much larger than themselves due to cutting and sucking the prey body content after extracorporeal digestion (Mulder *et al.*, 2011). Dorylaimid and aphelenchid predators with puncturing–sucking and piercing–sucking types, respectively, ingest prey body contents by sucking after extracorporeal digestion and thus may also not be dependent on their prey size in prey preference. In the diplogasterid predator *Mononchoides gaugleri*, no-choice and paired-choice predator–prey experiments showed that small and/or inert endoparasitic plant nematodes such as *Meloidogyne incognita*, *Heterodera mothi* and *Anguina tritici* juveniles were highly preferred as prey compared to large, rapid, endoparasitic or ectoparasitic plant nematodes, which generally holds true for all predatory nematodes except mononchids with a high level of aggressiveness and low prey specificity (Bilgrami *et al.*, 2005). This indicates that *M. gaugleri* shows moderate prey preference (with neither excessively wide nor narrow prey ranges) akin to other diplogasterid predators that have omnivorous feeding behaviours (Bilgrami and Jairajpuri, 1989a) and entomopathogenic nematodes such as *Steinernema carpocapsae* (Gaugler *et al.*, 1997). Dorylaimids have omnivorous feeding behaviours, and likewise, they can feed on a wide range of plant-parasitic nematodes (Russell, 1986). As in diplogasterid predators, dorylaimids have moderate feeding preferences on plant-parasitic nematodes (Bilgrami, 1992); however, they have advantages as effective biological control agents over mononchids in their species diversity, abundance and ubiquitous distribution in soil habitats (to have higher probabilities of encountering prey nematodes) but are at a disadvantage in terms of their long life cycle and low reproduction rates (also high sensitivity to environmental disturbances) (Bongers and Bongers, 1998). The feeding behaviour and fecundity of an aphelenchid predator (*S. paratenuicaudata*) vary depending upon its prey species; it feeds and reproduces continuously on mushroom-feeding (myceliophagous) nematodes such as *Aphelenchus avenae*, *Apehlenchoides* spp. and *Ditylenchus myceliophagus*, and small and/or inert endoparasitic plant nematode juveniles such as *M. incognita*, *Heterodera cajani*, *A. tritici* and *Subanguina chrysopogoni*, but not large and rapid predatory nematodes such as *Mylonchulus* species (Vats *et al.*, 2004), which also shows similar feeding preferences to those of diplogasterids and dorylaimids. *Seinura* species are not as aggressive as other predatory nematodes because of their weak armament (fine and weak needle-like style), indicating relatively low strike rates, which may be enhanced by the injection of toxic substance(s), leading to rapid paralysis of the prey nematodes and increased strike rates.

17.2.3 Mass production

Mass production and application of natural enemies is a prerequisite for the successful development and commercialization of biological control agents in terms of control and cost efficiencies because a very large population of predators should be released to successfully control plant-parasitic nematode species. This is one of the most inferior traits of predatory nematodes among other biological control agents because of their biotrophic nature (requiring current prey culture and rearing *in vivo*), relatively long life cycles, slow reproduction rates and low stability when compared with other microbial biological control agents such as fungi and bacteria. These inferior traits greatly reduce the efficiency of their mass culture and production. However, some variations exist in their production by culturing among feeding types of predatory nematodes, although they feed on not only prey nematodes but also other soil microorganisms, showing omnivorous feeding habits in general (Yeates *et al.*, 1993). Diplogasterids can be cultured on either prey nematodes or bacteria since they are facultative and biphasic (Bilgrami, 2008). Growth and development of diplogasterid predators occur by the support of both prey nematodes (*A. avenae*) and bacteria on a dixenic culture of the nematode and bacteria (Pillai and Taylor, 1968). Diplogasterid predators were cultivated

on agar plates using a bacterium *Bacillus cereus* var. *mycoides* or free-living nematode *Rhabditis* sp. supplemented with infant milk powder under *in vitro* conditions (Yeates, 1969; Bilgrami and Jairajpuri, 1988a). Dorylaimid predators can also be cultured using the same techniques mentioned above (Khan *et al.*, 1991, 1994, 1995a,b; Khan and Jairajpuri, 1997). However, mononchid predators are difficult to culture because of the culture of juvenile mononchids that cannot survive on the bacteria or agar alone, requiring possibly dead or wounded prey that have been attacked by the adults, disabling the ability for them to be cultured on a large scale. Adult mononchids feed randomly on nematode prey in culture and are easily maintained *in vitro* with plant-parasitic or free-living nematodes as prey (Cobb, 1917; Nelmes, 1974; Grootaert and Maertens, 1976; Mankau, 1980; Yeates, 1987a; Salinas and Kotcon, 2005). Aphelenchid predators (*Seinura* spp.) can be reared on fungal- and bacterial-feeding nematodes (Wood, 1974) and can reproduce on several mushroom-feeding nematodes and endoparasitic plant nematodes, in which case the reproduction rate should be high due to their short life cycles (Vats *et al.*, 2004). The growth and reproduction of *Seinura* spp. on fungi have rarely been examined, although many aphelenchids are fungivores that parasitize mushrooms and mycorrhizae and both grow and reproduce on fungal cultures.

17.2.4 Longevity and stability

Significant longevity and stability are required for predatory nematodes to be stored with no appreciable loss of their predation ability, and the development of BCA products with a long shelf-life (with biological control activity similar to the fresh products) is one of the main obstacles in the commercialization of BCAs (Bilgrami, 2008; Droby *et al.*, 2009; Coulibaly *et al.*, 2010). Little research has been conducted on the formulations of predatory nematodes of plant-parasitic nematodes for their storage with a stable shelf-life; however, extensive studies have been done on these matters for entomopathogenic nematodes. Vital factors for the long-term survival of infective juveniles of entomopathogenic nematodes and extending their shelf-lives were examined and applied to their formulations, for which sponge and vermiculite substrate were used to contain actively moving nematodes because of the full moisture contents preserved in these materials (requiring refrigeration during storage and transport) (Grewal, 2002). Alginate gels or liquid concentrates were developed to reduce nematode mobility because of their required spatial confinement (Kaya and Nelson, 1985; Kaya *et al.*, 1987; Georgis, 1990; Grewal, 1998). More efficient formulations suggested for long-term storage are those that reduce the metabolism of stored nematodes to partial anhydrobiosis through slow desiccation (Kondo and Ishibashi, 1989), which has been realized using anhydrous polyacrylamide gels, powders, granules and water-dispersible granules (Capinera and Hibbard, 1987; Connick *et al.*, 1993; Bedding and Butler, 1994; Georgis *et al.*, 1995; Silver *et al.*, 1995; Grewal and Georgis, 1998; Grewal, 2000a,b). The infective juveniles of the entomopathogenic nematode *Steinernema feltiae* survived in vermiculite for approximately 10 days, in alginate gels for 1 month, in water-dispersible granules for 2 months and in a wettable powder for 3 months (Grewal, 2000a). Recently, Chen and Glazer (2005) showed that calcium-alginate granulation of nematode solution with a high osmotic potential (composed of 18% glycerol) maintained the infectivity of entomopathogenic nematodes for 6 months (as with the freshly reared nematodes) due to the near anhydrobiotic state of the nematodes driven by the high osmotic potential. All of these aspects suggest that the long-term storage of entomopathogenic nematodes can be extended even further if the survival strategies of the nematodes such as quiescence (anhydrobiosis, anoxybiosis and cryobiosis) and cryptobiosis induce their physiological slow-down and shut-down under adverse environmental conditions (Freckman and Womersley, 1983; Wharton, 1986, 1995). This can be also applied to the long-term storage of predatory nematodes having several common characteristics in trophic type and feeding behaviour to entomopathogenic nematodes, especially for diplogasterid predators that form dauer

larvae similar to well-known entomopathogenic nematodes such as *Heterorhabditis* and *Steinernema* spp. in the order Rhabditida (Riddle *et al.*, 1981; Bongers and Bongers, 1998).

17.2.5 Easy application

The biological attributes of the predatory nematodes mentioned above such as mass production, longevity and stability are prerequisites for their usability in their easy application in the biological control of plant-parasitic nematodes. Low costs and compatibility with existing (standard) farm practices and agrochemicals are also useful characteristics of BCAs, which has been little documented for predatory nematodes. The following studies represent some portions of research done on such predatory nematode applications that reveal the current status of their application in the control of plant-parasitic nematodes. Khan and Kim (2005) cultured the diplogasterid predator *M. fortidens* on soil extract agar using *Paragrellus redivivus* as prey, and applied the predatory nematodes in different numbers after their extraction from the cultures using a Baermann funnel for the control of the root-knot nematode *Meloidogyne arenaria* in potted field soil. A significant reduction in the population density of *Globodera rostochiensis* and *M. incognita* was also observed in the presence of mononchid predator *Prionchulus punctatus* in pots (Small, 1977, 1979). These two studies suggest that the cultures of predatory nematodes reared on their prey nematodes can be applied in small scales to control plant-parasitic nematodes. However, the reliability of predatory nematodes in their applications under natural conditions is not as high as that in many other biological control agents that show decreased and inconsistent control efficacies in fields compared to those in pots or environment-controlled plots, although a significant negative correlation was found between the population densities of predatory nematode and plant-parasitic nematodes under field conditions (Ahmad and Jairajpuri, 1982). On the other hand, the omnivorous feeding habits of these predatory nematodes can be applied under natural conditions to boost their population densities by supporting fungal and bacterial growth using soil amendments such as the addition of chipped leaves, green manure and organic material into the soil (Linford and Oliviera, 1937; Lal *et al.*, 1983; Akhtar and Mahmood, 1993; Akhtar, 1995), which is assumed to be due to the increased availability of free-living nematodes due to the soil amendments and/or by the improved soil texture favoured by predatory nematodes with large body size such as mononchids and dorylaimids (Yeates, 1987b; Bouwman and Zwart, 1994; Ferris *et al.*, 1996; McSorley and Gallher, 1996; Bongers and Ferris, 1999; McSorley and Frederick, 1999). These cultural practices are greatly valuable for the predatory nematodes with difficulties in mass production and application to assist in the practical biological control of plant-parasitic nematodes under field conditions. For this application, the ecological features of predatory nematodes should be understood in detail.

17.3 Biocontrol Potentials of Predatory Nematodes

17.3.1 Mononchid predators

The mononchids belonging to the order Mononchida (sister order of Dorylaimida) are armed with one tooth, large puncturing teeth and/or numerous small grasping denticels in a strongly sclerotized buccal cavity (Fig. 17.2a). The capturing and feeding mechanisms of the mononchid predators are composed of an encounter with prey (by chance) after random movement, attack response (probing for selection and sensing the acceptable contour and texture of feeding sites) and attack by puncturing, mostly engulfing (swallowing) their prey or ingesting it after shredding it into pieces as in *Iotonchus*, but exceptionally cutting and sucking to feed on their prey as in *Mylonchulus* (Bilgrami *et al.*, 1986; Bilgrami, 2008). They swallow their prey by pulling it into their buccal cavity supported by the contractions of the oesophageal musculature. No mononchid predators are capable of

intentional movement in response to kairomones emitted by the prey, which is known as a way of encountering prey in diplogasterid, dorylaimid or nyglolaimid predators (Bilgrami and Jairajpuri, 1988a; Bilgrami and Pervez, 2000; Bilgrami *et al*., 2000, 2001).

The mononchid predators feed on all types of nematodes besides rotifers and other soil microorganisms. They are large nematodes with low reproduction rate and a high coloniser–persister (*c-p*) value (*c-p* 4) and relatively long life cycles with varying degrees depending on the species (Bongers *et al*., 1995). In order to complete one generation, 45 days are required in *P. punctatus* and 15 days are needed in *M. aquaticus* at 25°C (Maertens, 1975; Grootaert and Maertens, 1976). They occur in low densities in agricultural fields, especially under environmental stress, and in soils unrecovered from previous ecosystem disturbances through cultivation practices such as fertilization and tillage (Freckman and Caswell, 1985; Yeates and Bongers, 1999).

Despite some of the drawbacks to using mononchids as BCAs mentioned above, they have been considered as prospective predatory nematodes for reducing populations of plant-parasitic nematodes owing to their large body size, ability to be seen readily, high frequencies and distribution, as well as their strong external appearance with a strong armament consisting of a well-developed buccal cavity and musculature, teeth and denticles. Extensive studies have been conducted, and over 50 species of mononchids are currently reported as predators of a wide variety of plant-parasitic nematodes, which includes ten mononchid genera in the three families, Anatonchidae, Mononchidae and Itonchidae (Table 17.1). Of all the mononchids listed in Table 17.1, the genus *Iotonchus* has the most numerous (16) species, while *Coomansus* has the least with only one species. The predatory potential of mononchids has been extensively studied through observations of: predation (Nelmes, 1974; Small and Grootaert, 1983; Bilgrami *et al*., 1984; Kulshreshtha *et al*., 1993); predator strike rate, prey resistance and susceptibility to predation (Bilgrami and Jairajpuri, 1989a; Bilgrami, 1992, 1995); relationships with prey trophic groups (Bilgrami, 1992); range of prey (Small, 1979, 1987); cannibalism (feeding on conspecific individuals) (Bilgrami and Jairajpuri, 1984); and factors influencing predation (Bilgrami *et al*., 1983). Bilgrami *et al.* (1986) by analysing gut content in the intestine of the predators revealed that several commonly occurring mononchids feed extensively (though not exclusively) on different species of plant-parasitic nematodes and free-living nematodes, which suggested no or little prey resistance. However, this study also showed that more predators (75%) contained free-living nematodes in their intestine than tylenchs (45%) and dorylaims (41%) involving plant-parasitic nematodes. Yeates and Wardle (1996) suggested that mononchid nematodes predominantly prey on bacterial-feeding nematodes rather than plant-parasitic nematodes in addition to their capability of using bacterial food sources, which is either due to a prey preference toward free-living nematodes or due to their widespread occurrence, but the correct cause has not been determined experimentally to date.

According to Ahmad and Jairajpuri (1982), *Parahadronchus shakili* appears to be the most active predator of all the mononchids as 68% of the mononchid specimens contained prey in their intestine, including 24 genera of plant-feeding, free-living and even predatory nematodes, while *Coomansus* was the least active containing only 21% prey, suggesting variable predatory potentials depending upon the species of mononchid predators. They found a significant negative correlation between populations of the predatory nematode *P. shakili* and plant-parasitic nematodes. A significant negative correlation was also found between the populations of the mononchid predator *Iotonchus monhistera* and the plant-parasitic nematode *Helicotylenchus dihystera* (Azmi, 1983), and the existence of a predator–prey relationship was observed between the predator *Iotonchus tenuicaudatus* and the two plant feeders *Tylenchulus semipenetrans* and *H. dihystera* (Rama and Dasgupta, 1998). Similarly, several laboratory, pot and field experiments have shown the possibility of using mononchid predators for the biological control of the plant-parasitic nematodes, which was proposed first by Cobb (1917), e.g. *Clarkus papillatus* for the control of *Meloidogyne* sp.

Table 17.1. List of mononchid predators and their prey nematodes.

Predator family	Predator species	Prey nematodes	References
Anatonchidae	*Anatonchus amicae*	*Tylenchus*, *Xiphinema*	Coomans and Lima (1965)
c-p 4[a]	*A. ginglymodontus*	*Meloidogyne hapla* (juv.)	Szczygiel (1966, 1971)
	A. tridentatus	*Aglenchus agricola*, *Globodera rostochiensis*, *Longidorus*, *Paratylenchus macrophalus*, *Pratylenchus*	Mulvey (1961); Banage (1963)
	Miconchus aquaticus	*Hoplolaimus*, *Hemicyliophora*, *Xiphinema*	Bilgrami *et al.* (1986)
	M. citri	*Aphelenchoides*, *Pratylenchus*, *Tylenchorhynchus*	Bilgrami *et al.* (1986)
	M. dalhausiensis	*Aphelenchoides*, *Diphtherophora*	Bilgrami *et al.* (1986)
Itonchidae (*c-p* 4)[b]	*Itonchus acutus*	*Rotylenchus robustus*, *Trichodorus obtusus*, *X. americanum*	Cobb (1917), Thorne (1932)
	I. amphigonicus	*Heterodera schachtii*	Thorne (1924)
	I. antidontus	*Tylenchorhynchus*	Bilgrami *et al.* (1986)
	I. basidontus	*Tylenchorhynchus*	Bilgrami *et al.* (1986)
	I. brachylaimus	*Radopholus similis*, *Subanguina radicicola*	Cassidy (1931)
	I. kheri	*Helicotylenchus multicinctus*, *Hirshmanniella oryzae*, *M. incognita*, *Rotylenchulus reniformis*, *Scutellonema curvata*	Mohandas and Prabhoo (1980)
	I. longicaudatus	*Hirschmanniella*, *Hoplolaimus*	Bilgrami *et al.* (1986)
	I. monhystera	*H. multicinctus*, *Helicotylenchus dihystera*, *Heterodera oryzae*, *Hoplolaimus*, *M. incognita*, *Pratylenchus*, *Tylenchorhynchus nudus*, *R. reniformis*	Mohandas and Prabhoo (1980); Azmi (1983)
	I. nayari	*H. multicintus*, *H. oryzae*, *M. incognita*, *R. reniformis*, *X. elongatum*	Mohandas and Prabhoo (1980)
	I. parabasidontus	*H. oryzae*	Bilgrami *et al.* (1986)
	I. prabhooi	*M. incognita*, *R. reniformis*	Mohandas and Prabhoo (1980)
	I. risoceiae	*Pratylenchus*	Bilgrami *et al.* (1986)
	I. shafi	*Hoplolaimus*	Bilgrami *et al.* (1986)
	I. tenuicaudatus	*Tylenchulus semipenetrans*, *H. dihystera*	Rama and Dasgupta (1998)
	I. trichurus	*Hoplolaimus*, *Pratylenchus*, *Tylenchorhynchus*, *Xiphinema*	Bilgrami *et al.* (1986)
	I. vulvapapillatus	*Tylenchorhynchus*	Andrássy (1964)
Mononchidae *c-p* 4	*Clarkus mulveyi*	*H. multicinctus*, *M. incognita*, *R. reniformis*, *T. nudus*	Mohandas and Prabhoo (1980)
	C. papillatus	*Aphelenchoides*, *Hemicroconemoides*, *H. schachtii*, *M. hapla*, *Tylenchus*, *T. semipenetrans*, *S. radicicola*	Cobb (1917); Menzel (1920); Steiner and Heinly (1922); Thorne (1927); Szczgiel (1966, 1971)
	C. sheri	*Tylenchorhynchus*	Bilgrami *et al.* (1986)

Continued

Table 17.1. Continued.

Predator family	Predator species	Prey nematodes	References
	C. venezolanus	*Helicotylenchus*	Loof (1964)
	Coomansus indicus	*Hemicriconemoides*, *Pratylenchus*	Bilgrami *et al.* (1986)
	Mononchus aquaticus	*Anguina tritici*, *G. rostochiensis*, *H. oryzae*, *H. indicus*, *H. mothi*, *Longidorus*, *M. incognita*, *M. naasi*, *Paralongidorus citri*, *Paratrichodorus*, *Rotylenchus fallorobustus*, *Trichodorus*, *Tylenchorhynchus mashhoodi*, *X. americanum*	Grootaert and Maertens (1976); Grootaert and Wyss (1979); Small and Grootaert (1983); Bilgrami (1992)
	M. truncatus	*H. schachtii*	Thorne (1927)
	M. tunbridgensis	*Hemicriconemoides*, *Hoplolaimus*, *Tylenchorhynchus*	Mankau (1980) Bilgrami *et al.* (1986)
	Mylonchulus agilis	*Helicotylenchus vulgaris*, *L. caespiticola*, *R. fallorobustus*	Doucet (1980)
	M. brachyuris	*S. radicicola*, *R. similis*	Cassidy (1931)
	M. dentatus	*A. tritici*, *Basiria*, *Helicotylenchus indicus*, *H. oryzae*, *Hoplolaimus indicus*, *Longidorus*, *M. incognita*, *P. citri*, *T. mashhoodi*, *T. semipenetrans*, *X. basiri*	Jairajpuri and Azmi (1978); Bilgrami and Kulshreshtha (1994)
	M. hawaiiensis	*H. oryzae*, *M. incognita*, *T. nudus*, *R. reniformis*	Mohandas and Prabhoo (1980)
	M. minor	*A. tritici*, *M. incognita*, *T. semipenetrans*, *X. americanum*, *R. reniformis*	Kulshreshtha *et al.* (1993); Choudhari and Sivakumar (2000)
	M. parabrachyuris	*H. schachtii*	Thorne (1927)
	M. sigmaturus	*H. schachtii* (eggs), *M. javanica*, *R. similis*, *S. radicicola*, *T. semipenetrans*	Thorne (1927); Cassidy (1931); Cohn and Mordechai (1973, 1974)
	Prionchulus muscorum	*Aphelenchoides*, *Hemicriconemoides*, *Hololaimus*, *Tylenchorhynchus*	Altherr (1950); Szczygiel (1971); Arpin (1976); Bilgrami *et al.* (1986)
	P. punctatus	*A. tritici*, *G. rostochiensis*, *H. dihystera*, *M. naasi*, *R. fallorobustus*	Small and Evans (1981); Small and Grootaert (1983)
	Sporonchuls ibitensis	*Aphelenchoides*	Carvalho (1951)
	S. vagabundus	*Aphelenchoides, Pratylenchus, Hemicycliophora, Trichodorus*	Bilgrami *et al.* (1986)

[a]Colonizer–persister (*c-p*) values (Bongers, 1990).
[b]Presumed *c-p* value

(Cobb, 1920; Steiner and Heinly, 1922), the usefulness of predatory nematodes in the control of plant-parasitic nematodes (Cassidy, 1931; Christie, 1960; Mulvey, 1961; Esser, 1963; Esser and Sobers, 1964; Ritter and Laumond, 1975), a constant correlation of a low citrus nematode population with a high *Mylonchulus sigmaturus* population level in pot experiments (Cohn and Mordechai, 1974) and significant reductions in populations of potato cyst nematode, *G. rostochiensis* and root-knot nematode, *M. incognita* in the presence of a predatory nematode, *P. punctatus* (Small, 1979).

Contrary to the above studies providing positive evidence and explanations for the possibility of using mononchid predators as BCAs of plant-parasitic nematodes, several studies have also indicated their low possibilities as biocontrol agents of plant-parasitic nematodes. Thorne (1927) found that the soil population of mononchids present in sugarbeet fields tended to be unstable and to change rather abruptly, sometimes disappearing or becoming drastically reduced, suggesting a sceptical view of the biocontrol potential of these mononchids. Webster (1972) and Jones (1974) indicated that nonspecific predators like mononchids might come to exert only partial control of plant-parasitic nematodes. Most of all, flaws in the mononchid predators from a practical biocontrol standpoint do not rely on their predation capacity, especially predation rate with a low resistance from their prey, but rather on their biological and ecological characteristics such as a low rate of reproduction, long life cycle, sensitivity to environmental stress and cannibalism (Khan and Kim, 2007). Low specificity to the target prey may also attenuate their potential for the biocontrol of plant-parasitic nematodes.

17.3.2 Diplogasterid predators

Diplogasterids belonging to the order Diplogasterida are characterized by movable teeth arming the buccal cavity that is relatively smaller than in mononchids (Fig. 17.2b). They are predators, omnivores and bacterial and fungal feeders, feeding on nematodes, bacteria and other soil microorganisms. Diplogasterids are all enrichment opportunists with short life cycles (scaled *c-p* 1), completing their life cycles in 8–15 days and can be cultured and maintained on simple nutrient media containing bacteria as a food source (Yeates, 1969; Yeates and Bongers, 1999). Around nine diplogasterid species in four genera (two families) have been recorded as predators of plant-parasitic nematodes, including *Butlerius degrissei*, *Butlerius micans*, *Fictor anchicoprophaga*, *Mononchoides bollingeri*, *Mononchoides changi*, *M. fortidens*, *M. gaugleri*, *M. longicaudatus* and *Odontopharynx longicaudatus* (Table 17.2). No plant-parasitic nematodes have been reported as prey for *Diplonteron colobocercus* and *Mononchoides composticola* (Yeates, 1969; Steel *et al.*, 2011), although their feeding behaviour and predation abilities on saprozoic nematodes enlightened the general characteristics of diplogasterid predators in relation to the biological control of plant-parasitic nematodes. Among the diplogasterid predators mentioned above, four species including *B. micans*, *F. anchicoprophaga*, *M. bollingeri*, and *M. changi* are to be also excluded as predators of plant-parasitic nematodes because only *A. avenae* is recorded as their prey, meaning that it is not a plant-parasitic but rather fungivorous nematode (Rhodes and Linford, 1959; Kim, 1994; Jun and Kim, 2004). However, a strong possibility exists that these diplogasterid predators would have feeding abilities on plant-feeding nematodes in further extensive experimentations provided with various nutritional and environmental conditions. No prey mechanisms specifically excluding plant parasites from other trophic types of nematodes (or vice versa) have been found in any diplogasterid predators. In this respect, *M. composticola* may have good potential to be developed as a useful BCA of plant-parasitic nematodes because this predatory nematode has the biological control characteristics of selective predation, high predation rate, active moving toward prey and dual feeding behaviour for bacterial and nematode prey, suggesting its high control capacity and persistence in agricultural soil ecosystems (Steel *et al.*, 2011).

Among the diplogasterid predators listed in Table 17.2, three *Mononchoides* species (*M. fortidens*, *M. gaugleri* and *M. longicaudatus*) and *Odontopharynx longicaudatus* were more extensively studied based on their feeding behaviour on plant-parasitic nematodes than other diplogasterids, and thus they would be the most promising candidates for the biological control of plant-parasitic nematodes.

The prey catching and feeding mechanisms of *M. longicaudatus* and *M. fortidens* consist of five more or less distinct phases, i.e. encounter with prey (lip contact of predators with prey) after their random movement, the probing of prey (attack response), attack by predators after sensing the acceptable contour

Table 17.2. List of diplogasterid predators and their prey nematodes.

Predator family	Predator species	Prey nematodes	References
Diplogasteridae *c-p* 1[a]	*Butlerius degrissei*	*Aphelenchus avenae*, *Aphelenchoides fragariae*, *Pratylenchus*, *G. rostochiensis* (juv.), *R. robustus*	Grootaert *et al.* (1977); Small and Grootaert (1983)
	B. micans	*A. avenae*	Pillai and Taylor (1968)
	Fictor anchicoprophaga	*A. avenae*	Pillai and Taylor (1968)
	Mononchoides bollingeri	*A. avenae*	Goodrich *et al.* (1969)
	M. changi	*A. avenae*	Goodrich *et al.* (1969)
	M. fortidens	*M. incognita* (juv.), *A. tritici* (juv.), *M. arenaria*	Bilgrami and Jairajpuri (1988a, 1989a); Khan and Kim (2005)
	M. gaugleri	*M. incognita* (juv.), *A. tritici* (juv.), *H. mothi* (juv.), *Tylenchorhynchus mashhoodi*, *Longidorus*, *X. americanum*, *Trichodorus*, *H. indicus*, *H. mangiferae*, *P. christei*	Bilgrami *et al.* (2005)
	M. longicaudatus	*M. incognita* (juv.), *A. tritici* (juv.), *T. mashhoodi*, *X. americanum*, *H. indicus*, *Longidorus*, *Trichodorus*	Bilgrami and Jairajpuri (1988b, 1989b)
Odontopharyngidae *c-p* 1	*Odontopharynx longicaudata*	*A. amsinckiae*, *A. pacificae*, *A. gragariae*, *Criconemella xenoplax, M. hapla, M. incognita, M. javanica, P. vulnus, Trichodorus, T. semipenetrans, X. index*	Chitambar and Noffsinger (1989)

[a]Colonizer–persister (*c-p*) values (Bongers, 1990).

and texture of the prey cuticle, salivation/extracorporeal digestion (release of oesophageal secretions) and the intake of prey contents by ingestion/feeding (Bilgrami and Jairajpuri, 1989a). The predation rates by adults of the above two *Mononchoides* species are high (9–22 nematodes per day for 12 days) for plant-parasitic nematodes except for the large ectoparasitic nematodes *Hemicriconemoides mangiferae* and *Hoplolaimus indicus*.

Three *Mononchoides* (*M. fortidens*, *M. gaugleri* and *M. longicaudatus*) have high degrees of prey preference towards small and weak nematodes, such as choosing the juveniles of endoparasitic nematodes over large and strong ectoparasitic nematodes (Bilgrami and Jairajpuri, 1988a; Bilgrami *et al.*, 2005). This feeding preference is related to the resistance of prey nematodes to predation, and to the strike rates of predators. It was discovered that second-stage juveniles (J_2) of the plant-parasitic nematodes *M. incognita* and *A. tritici* were most susceptible to predation, but that *Hemicriconemoides mangiferae* and *H. indicus* were completely resistant to predation, despite the fact that *M. longicaudatus* and *M. fortidens* attack all kinds of prey (Bilgrami and Jairajpuri, 1989a,b). Fauzia *et al.* (1998) and Khan and Kim (2005) showed that *M. longicaudatus* and *M. fortidens*, respectively, reduced root galling on plants in pot tests, resulting in increased vegetative growth and root mass, and the suppression of the root-knot nematode populations, respectively.

In the latter experiment, the final population density of *M. fortidens* increased by approximately two-fold in comparison with initial populations of the predatory nematode. These results may be due to the preferential feeding behaviour of diplogasterid predators toward endoparasitic nematodes. *O. longicaudatus* is also prey-specific as it attacked and killed 6 out of 17 prey nematode species presented in the laboratory study, and in particular eliminated an entire population of *Anguina pacificae*, suggesting that a target prey–predator relationship may exist in the field habitat where the two nematode species were collected (Chitambar and Noffsinger, 1989). Selective feeding of the predatory nematodes on specific plant-parasitic nematodes may increase their control efficacy because of the increased strike rate on target prey.

Yeates (1969) and Grootaert *et al.* (1977) found that *Diplenteron colobocercus* and *Butlerius degrissei*, respectively, switch over to feed on bacteria in the absence of prey nematodes, suggesting their enhanced capability for persistence when prey nematode populations are reduced. Other diplogasterid predators mentioned above may also be able to persist in varied soil environments, an attribute that is derived from their dual feeding behaviour for bacterial and nematode prey and their rapid colonizing ability due to a high reproduction rate (Bongers *et al.*, 1995). These characteristics of feeding behaviour are another desirable feature of diplogasterid predators in terms of the biological control of plant-parasitic nematodes; by maintaining predator population densities, a consistent rate of control is exerted over the parasites.

As noted above, diplogasterid predators have various traits that make them valuable BCAs of plant-parasitic nematodes. The main advantages of diplogasterids lie in their chemotaxis sense (increasing encounters with prey nematodes), high predation rate, prey specificity, ease of culture and persistence under adverse conditions (Bilgrami and Jairajpuri, 1990). Further research on new diplogasteirds with, for example, highly advanced biological control traits, mass culturing techniques and control efficacy in the field, will be required to confirm their usefulness in the control of the plant-parasitic nematodes.

17.3.3 Dorylaimid predators

Nematodes in the order Dorylaimida are mostly free-living predators except for the plant-parasitic nematode families Longidoridae (*Longidorus*, *Paralongidorus* and *Xiphinema*) and Trichodoridae (*Trichodorus* and *Paratrichodorus*) in the class Adenophorea. They have a piercing–sucking-type of feeding using a hollow feeding apparatus called an odontostyle (in the case of dorylaimid predators) (Fig. 17.2c) or a large protrusible tooth called a mural tooth (in the case of nyglolaimid and trichodorid predators), which is used to pierce or slit the bodies of their prey before sucking out the contents (Bilgrami and Gaugler, 2004). They have omnivorous feeding characteristics, and thus can switch to feed on other soil microorganisms such as bacteria and fungi and even algae (Wyss and Grootaert, 1977; Russell, 1986; Shafqat *et al.*, 1987; Khan *et al.*, 1991). These omnivorous feeding characteristics may enhance their survival in the event of a lack of prey nematodes and other food sources.

Most nematodes in the order Dorylaimida have long life cycles that vary depending on genera and species, generally taking 3–6 months, but as little as 36 days in the case of *Labronema vulpapillatum* with *c-p* groups 4 and 5, for which their populations tend to diminish as soon as their habitats are altered (Ferris, 1968; Wood, 1973; Johnson *et al.*, 1974; Zullini, 1976; Bongers *et al.*, 1995; De Ley and Blaxter, 2002). However, dorylaimids are presumed to be the most diverse and abundant group among soil and freshwater nematodes and the most ubiquitous group of predatory nematodes with their abundance to be estimated at 200–500 million/acre (De Ley and Blaxter, 2002). They occur in all types of soils, climates and habitats (Mullin, 2004).

Fourteen species have been recorded as dorylaimid predators preying on plant-parasitic nematodes, of which the families (genera) are Qudsianematidae (*Allodorylaimus*, *Labronema*, *Eudorylaimus*, *Mesodorylaimus*), Discolaimidae (*Discolaimus*), Dorylaimidae (*Dorylaimus*), Nordidae (*Thornia*), Nyglolaimidae (*Aporcelaimellus, Auatides*) and Trichodoridae (*Westindicus*) (Table 17.3).

Table 17.3. List of dorylaimid predators and their prey nematodes.

Predator family	Predator species	Prey nematodes	References
Discolaimidae	*Discolaimus arenicolus*	*Meloidogyne incognita* (juv.)	Yeates *et al.* (1993)
c-p 5[a]	*D. silvicolus*	*M. incognita* (juv.), *H. mothi* (juv.), *A. tritici* (juv.), *X. basiri*, *Longidorus*, *T. mashoodi*, *H. oryzae*, *Aphelenchoides*, *Basiria*, *A. avenae*, *T. semipenetrans*, *Trichodorus*	Khan *et al.* (1995a)
Dorylaimidae	*Dorylaimus obscurus*	*H. schachtii* (eggs)	Thorne and Swanger (1936)
c-p 4	*D. stagnalis*	*T. mashoodi*, *H. oryzae*, *H. indicus*, *X. americanum*, *Longidorus*, *P. citri*, *A. tritici* (juv.), *M. incognita* (juv.), *H. mothi* (juv.)	Bilgrami (1992); Shafqat *et al.* (1987)
Nordidae *c-p* 4	*Thornia*	*Criconemoides*, *Meloidodera floridensis*, *P. curvitatus*, *P. penetrans*, *P. vulnus*, *T. semipenetrans*	Boosalis and Mankau (1965); Esser (1987)
Nygolaimidae *c-p* 5	*Aporcelaimellus nivalis*	*A. tritici*, *Aphelenchoides*, *Basiria*, *H. indicus*, *H. mangiferae*, *H. dhirenderi*, *H. moothi*, *H. oryzae*, *H. indicus*, *Longidorus*, *M. incognita*, *P. citri*, *Scutellonema*, *T. mashhoodi*, *T. semipenetrans*, *Trichodorus*, *X. americanum*, *X. insigne*	Khan *et al.* (1991); Bilgrami (1993)
	A. obscurus	*H. schachtii* (juv.)	Thorne and Swanger (1936)
	A. obtusicaudatus	*H. schachtii* (juv.)	Marinari *et al.* (1982)
	Aquatides thornei	*A. tritici*, *H. indicus*, *H. mothi*, *H. oryzae*, *Longidorus*, *M. incognita*, *P. citri*, *T. mashhoodi*, *Paratrichodorus*, *X. americanum*	Bilgrami *et al.* (1985); Bilgrami (1992)
Qudsianematidae *c-p* 4	*Allodorylaimus americanus*	*M. incognita* (juv.), *A. tritici* (juv.), *Xiphinema basiri*, *Longidorus*, *T. mashoodi*, *H. oryzae*, *Aphelenchoides*, *Basiria*, *A. avenae*, *T. semipenetrans*, *Trichodorus*	Khan *et al.* (1995a,b)
	Eudorylaimus obtusicaudatus	*H. schachtii*	Esser (1987)
	Labronema vulvapapillatum	*A. avenae*, *A. tritici* (juv.), *M. naasi* (juv.), *G. rostochiensis (juv.)*	Wyss and Grootaert (1977); Grootaert and Small (1982); Small and Grootaert (1983); Esser (1987)
	Mesodorylaimus bastiani	*M. incognita* (juv.), *H. mothi* (juv.), *X. basiri*, *X. americanum*, *X. insigne*, *Longidorus*, *T. mashoodi*, *H. oryzae*, *H. indicus*, *Aphelenchoides*, *Basiria*, *A. avenae*, *T. semipenetrans*, *Trichodorus*, *Paratrichodorus*, *A. tritici* (juv.)	Bilgrami (1992)
Trichodoridae *c-p* 4	*Westindicus rapax*	*Xiphinema*	Hunt (1978)

[a]Colonizer–persister (*c-p*) values (Bongers, 1990).

The predatory potential of each of these dorylaimids is known mostly from *in vitro* studies. In a family of the order Dorylaimida, the range of prey plant-parasitic nematodes varied depending on the species of dorylaimid predators, which determines their control potential over the plant-parasitic nematodes. For endoparasitic sedentary plant-parasitic nematodes, most dorylaimid predators (other than *Allodorylaimus amylovorus*, *Discolaimus arenicolus* and *Thornia* spp.) feed on either eggs or juveniles of one or more cyst nematode species (notably *Heterodera schachtii*), suggesting they have biocontrol potential of the cyst nematodes. In addition, approximately half of dorylaimid predators consume endoparasitic sedentary root-knot nematodes (primarily *M. incognita*) as their prey. When the semi-endoparasitic citrus nematode *T. semipenetrans* or ectoparasitic and virus-transmitting nematodes *Longidorus*, *Xiphinema*, *Trichodorus* and *Paratrichodorus* are attacked by one or more dorylaimid predators, their populations decline and/or reduced nematode damage to plants occurs. All of these aspects suggest that dorylaimid predators may have a wide range of prey among plant-parasitic nematodes (Table 17.3). They are attracted toward prey and aggregate at feeding sites in response to prey secretions, suggesting an efficient prey searching ability (Bilgrami and Pervez, 2000; Bilgrami *et al.*, 2000; Bilgrami and Gaugler, 2005). In addition, dorylaimids have reliable characteristics of nematode control such as efficient rates of predation (Khan *et al.*, 1991; Bilgrami, 1992) and a positive correlation between predation and prey trophic groups (Bilgrami, 1993, 1995). All of these aspects of dorylaimid predators, including their widespread and abundant presence, omnivorous feeding habits, ability to perceive prey kairomones and inverse relationships with prey populations observed in pot trials (Boosalis and Mankau, 1965), make them prime candidates for the biological control of plant-parasitic nematodes.

The nematode families in Dorylaimida have the highest *c-p* values among soil and freshwater nematodes, including Leptonchidae, Qudsianematidae and Trichodoridae, with *c-p* 4 and Aporcelaimidae and Longidoridae with *c-p* 5, making them vulnerable to soil disturbances and environmental stress such as the mononchid predators mentioned above (Bongers *et al.*, 1995; Yeates and Bongers, 1999). Biotic and abiotic factors such as temperature, density, starvation and incubation affect their chemotactic response (Bilgrami and Jairajpuri, 1988a), dispersion of prey kairomones (Green, 1980) and rate of predation (Bilgrami, 1995), resulting in changes in feeding and predatory activity, aggregation to feeding sites and prey search activities. Predatory activity was reduced and prey attractants depleted under experimental temperature extremes (Huettel, 1986; Bilgrami *et al.*, 2000). Dorylaimoid predators after 6 days of short-term food deprivation displayed enhanced predatory ability, and were able to detect more prey individuals due to a decreased minimum response threshold that enabled them to perceive weak stimuli from the prey much faster than their well-fed counterparts (Doncaster and Seymour, 1973). Similar to other groups of predators, dorylaimids showed density-dependent predation, since high prey densities may increase predator–prey encounters, resulting in an increased predation rate (Khan *et al.*, 1991). These biological and ecological characteristics of dorylaimid predators should be considered sufficiently to maximize their biocontrol efficiencies in the management and control of plant-parasitic nematodes.

17.3.4 Aphelenchid predators

Nematodes in the order Aphelenchida have a stylet for feeding and a very prominent median bulb in the oesophagus (Fig. 17.2d). They are cosmopolitan and associated with: (i) eukaryotic organisms such as insects as parasites or symbionts (phoronts); (ii) plants as root, stem or leaf parasites; (iii) fungi as fungivores; and (iv) other nematodes as predators with a considerable plasticity of feeding habits within a species sometimes depending on the immediate availability of different foods. Among them, species of *Seinura* in the family Aphelenchoididae (or Seinuridae) are predatory on other nematodes.

Seinura spp. have a fine needle-like stylet that penetrates the cuticle of prey nematodes

and injects digestive enzymes into the prey body to paralyse the prey instantly. This is followed by the sucking-out of the body contents so that they can feed on prey with a larger body size than their own (Linford and Oliviera, 1937; Hechler, 1963; Wood, 1974). *Seinura* spp. have life cycles of 3–6 days with *c-p* 2 and a high reproductive potential; they can be cultured rapidly on fungivorous nematodes under laboratory conditions. Presently, seven *Seinura* spp. are known to be predatory on various plant-parasitic nematodes including *T. semipenetrans*, juveniles of cyst nematodes (*Heterodera* spp.) and root-knot nematodes (*Meloidogyne* spp.), along with several myceliophagous nematodes (*Aphelencus* spp. and *Aphelenchoides* spp.) that feed on mushrooms (Table 17.4). Among them, *S. paratenuicaudata*, which is frequently encountered along with myceliophagous nematodes in mushroom houses, has a wide range of prey nematodes such as *S. tenuicaudata* (Linford and Oliviera, 1937; Hechler, 1963; Vats *et al*., 2004). It can feed and reproduce on ten plant-parasitic and mushroom-feeding nematode species. *S. paratenuicaudata* has a short life cycle of 4–5 days, and like other species of *Seinura*, it feeds on prey nematodes continuously from J_2 soon after hatching until becoming active-preying adult nematodes. A single J_2 can feed on prey for 1.5–2 h continuously, killing one to three nematodes before moulting, whereas adult nematodes feed on and kill 4–6 prey nematodes in a day, indicating a high predatory potential (Vats *et al*., 2004). However, *S. paratenuicaudata* feeds on no larval stages or adults of larger nematodes such *H. dihystera*, *H. indicus*, *Longidorus pisi* or J_2 and males of root-knot nematodes (*M. incognita* or *M. javanica*), *Heterodera avenae*, *Heterodera sorghi* or *Heterodera zeae*. It also feeds on, and reproduces with, the J_2 of *A. tritici* and *Subanguina chrysopogoni*, but the eggs produced are mostly infertile. *S. paratenuicaudata*'s feeding activity and reproduction vary depending on temperature, and it fails to feed or multiply between 11°C and 15°C. All of these aspects suggest that the aphelenchid predators of *Seinura* spp. have a high potential for the biological control of plant-parasitic nematodes because of their biological and predatory characteristics (high reproduction and predation

Table 17.4. List of aphelenchid predators and their prey nematodes.

Predator family	Predator species	Prey nematodes	References
Aphelenchoididae *c-p* 2[a]	*Seinura celeris*	*Aphelenchus avenae*	Hechler and Taylor (1966)
	S. demani	*A. avenae*, *A. bicaudatus*	Wood (1974)
	S. oliveriae	*A. avenae*	Hechler and Taylor (1966)
	S. oxura	*A. avenae*, *Ditylenchus myceliophagus*	Hechler and Taylor (1966); Cayrol (1970)
	S. paratenuicaudata	*A. tritici* (juv.), *Subanguina chrysopogoni* (juv.), *Aphelenchoides bicaudatus*, *A. composticola*, *A. avenae*, *A. radicicolus*, *Ditylenchus myceliophagus*, *Heterodera zeae* (juv.), *H. cajani* (juv.), *H. sorghi* (juv.), *Meloidogyne incognita* (juv.), *M. javanica* (juv.)	Vats *et al.* (2004)
	S. steineri	*A. avenae*	Hechler and Taylor (1966)
	S. tenuicaudata	*Meloidogyne marioni (juv.), Pratylenchus pratensis, A. avenae, A. parientinus, D. dipsaci, Heterodera trifoli* (juv.), *M. hapla* (juv.), *Neotylenchus linfordi*	Linford and Oliviera (1937); Hechler (1963)

[a]Colonizer–persister (*c-p*) values (Bongers, 1990).

rates, and easy mass culture), when applied to the correct target nematodes using proper management tactics to maximize their control efficiencies.

17.4 Conclusions and Future Prospects

Among the natural enemies and antagonists of plant-parasitic nematodes, the most extensive research efforts have been made on predaceous (trapping) and endoparasitic fungi, comprising over 73–76% of the total research efforts, followed by predatory nematodes at 7–13% (Kerry, 1987; Bilgrami, 2008). In terms of research efforts on predatory nematodes, their biological attributes in relation to control efficacy such as predation ability and prey searching capability have been more extensively studied than those related to their usability as BCAs such as mass production, longevity, stability (storage) and easy application.

The control efficacy of predatory nematodes is determined by the product of predation ability and prey searching capability, which is comparable to the real number of prey removed from the habitat through their predation on the prey encountered by the predators through their searching activity. Prey predation is composed of prey capturing and feeding, which is divided into the predation phases: encounter with prey, attack response and feeding (composed of attack, extracorporeal digestion and ingestion; Bilgrami and Jairajpuri, 1989b; Bilgrami, 2008). Mononchid predators belonging to the three families Anatonchidae, Iotonchidae and Mononchidae encounter prey nematodes through accidental contact, indicating a low probability of prey capture, while other predatory nematode groups such dorylaimids, diplogasterids and aphelenchids make intentional contact with prey nematodes in chemosensory responses to kairomones emitted by the prey, increasing the probability of predator–prey contacts (Grootaert and Maertens, 1976; Bilgrami and Jairajpuri, 1988a; Bilgrami, 1997; Bilgrami and Pervez, 2000; Bilgrami *et al.*, 2000, 2001; Pervez and Bilgrami, 2000). Mononchid predators show more aggressive attack responses and vigorous attacks using the feeding mechanisms of engulfing, generally leading to higher strike rates (SR: number of encounters resulting in attack/number of total encounters), higher prey susceptibility (PS: number of attacks resulting in wounding/number of total attacks), broader prey spectrum efficiency and shorter feeding time than the other three predatory nematodes. However, the predation abilities of mononchids are outweighed by their low prey capturing abilities that result from accidental predator–prey contact, such that their overall predation abilities are reduced below those of the other predatory nematodes. Mononchids show cannibalism (feeding on conspecific individuals), which further reduces their biological control potential (Bilgrami, 2008). Diplogasterids, dorylaimids and aphelenchids with the same ingestion mechanism of sucking after cutting using movable teeth, puncturing with an odontostyle or onchiostyle and piercing with a stomatostyle have similar SR and PS in varying degrees depending on the prey nematode species, showing relatively higher SR and PS against endoparasitic nematodes than ectoparasitic nematodes (even with no occurrence of attacks against several nematode species in the families of Hoplolaimidae and Criconematidae, which have a thick cuticle and strong annulation, respectively). *Seinura* species belonging to Aphelenchoididae (aphelenchid predators) attack their prey by injecting toxic substances for the immobilization of their prey, which is another attribute that increases the predation ability of a successful BCA (Bilgrami, 2008).

Another control efficacy-related attribute is searching capability, which can be determined by colonizing ability and persistence (or dispersal). Among these predatory nematodes, diplogasterids and aphelenchids have higher searching capabilities because of their high colonizing abilities (*c-p* 1 (enrichment opportunists) or *c-p* 2 (general opportunists) on the coloniser–persister (*c-p*) scale) due to their short generation time and high reproduction rates compared with mononchids or dorylaimids of *c-p* 4 or *c-p* 5 due to their long generation time and low fecundity (Bongers and Bongers, 1998). The enrichment opportunists, diplogasterids such as *Mononchoides*

species, result in explosive population growth under food-rich conditions, and can be practically applied for pest control by boosting population numbers using soil amendments of compost and manure (Steel *et al.*, 2011).

Seinura species in Aphelenchoididae are general opportunists that may be multiplied prominently in mushroom compost, and thus they can efficiently control mushroom-feeding nematodes such as *Aphelenchoides* spp. and *Ditylenchus myceliophagus* (Vats *et al.*, 2004). Mononchids and dorylaimids differ in their searching capabilities because of their persistence or dispersal – the former lowered by their confined distribution with poor species abundance and the latter increased by their wide distribution and high species abundance. However, the massive introduction of mononchids can be applied for intensive short-term control of plant-parasitic nematodes in heavily infested crops growing in confined areas such as glasshouses, due to their aggressive and vigorous feeding behaviours that make them suitable for pest control under such conditions of highly probable contact with prey due to the large numbers of the prey nematodes. In contrast, dorylaimids can be used for the long-term control of chronic nematode diseases using agricultural practices that maintain the populations of these resident predatory nematodes.

The usability of BCAs is related to their successful development and commercialization, which is determined mainly by their ease of mass production, longevity, stability and ease of application (Kerry, 1987). The culturing of predatory nematodes for mass production is the most feasible for diplogastrids due to their omnivorous behaviour, feeding on nematodes and bacteria, and having high reproduction rates. This culturing is the least feasible for mononchids due to their fastidious predaceous behaviour, feeding mostly on prey nematodes, and having low reproduction rates. Cultured growth is moderately feasible for dorylaimids and aphelenchids with both omnivorous behaviour and easy culture *in vitro*. Longevity and stability are related to the shelf-life (storage) of BCA products, which is one of the key criteria for their successful commercialization; however, this biological attribute has been studied little in predatory nematodes for the biological control of plant-parasitic nematodes. In entomopathogenic nematodes, however, vital factors for the long-term survival of their infective juveniles and the extension of their shelf-lives were examined and applied to their formulations. Strategies that were most effective in providing long-term storage were those that reduced the metabolism of stored nematodes to partial anhydrobiosis (dormant state) using slow desiccation techniques or calcium alginate-mediated high osmotic desiccation and various formulations such as anhydrous polyacrylamide gels, powders, granules, water-dispersible granules and wettable powder (Capinera and Hibbard, 1987; Kondo and Ishibashi, 1989; Connick *et al.*, 1993; Bedding and Butler, 1994; Georgis *et al.*, 1995; Silver *et al.*, 1995; Grewal and Georgis, 1998; Grewal, 2000a,b). These techniques and formulations can be applied in the commercialization of predatory nematodes, suggesting them as a feasible BCA, especially diplogasterids that share a common biological feature of forming dauer larvae (a type of stasis enabling them to survive harsh conditions) as typical entomopathogenic nematodes, such as *Heterorhabtidis* and *Steinernema* spp. in Rhabditida (Riddle *et al.*, 1981; Bongers and Bongers, 1998). The mass production of predatory nematodes with a long shelf-life is prerequisite for their easy application in the biological control of plant-parasitic nematodes, indicating an advantageous feature of diplogasterids, a disadvantageous feature of mononchids and a neutral feature for dorylaimids and aphelenchids. However, all predatory nematodes can be applied for pest control using practical approaches designed for their biological control attributes to obtain maximum control efficacy.

Considering the biological attributes of predatory nematodes in relation to control efficacy mentioned above, diplogasterids are best suited for the biological control of plant-parasitic nematodes due to their high levels of predation ability and prey searching capability (prey specificity and colonizing ability). Mononchids are least suited because of their low levels of predation ability, due mainly to accidental encounters with prey and low prey searching capabilities (colonizing ability and confined distribution). Dorylaimids and

aphelenchids each possess high and low degrees of these respective attributes and are therefore medially suited for nematode biological control. However, most of the information on specific biological attributes for control efficacy is based on agar plate experiments provided for several prey species, which is not sufficient to reveal fully their activities under natural conditions. More research needs to be conducted on the predation ability and searching capability of predatory nematodes, and investigations of the factors affecting predator–prey interactions *in situ* should be made to disclose their biological control potential in field to help determine optimum application strategies for maximum pest control efficacy. For this, not only do the eco-biological characteristics of predatory nematodes need to be known, but those of other components such as plant-parasitic nematodes and host plants also need to be examined. This research can reveal specific predator–parasite–host relations, which can be applied for the successful biological control of specific nematode diseases using predatory nematode, e.g. the timely application of predators concomitant to the emergence of juveniles of sedentary endoparasitic nematodes on crop roots. A few things also appear to be missing from the (mostly) agar plate experiments. One thing found in prey predation experiments (e.g. Bilgrami and Jairajpuri, 1989a,b; Bilgrami, 1992) was a lack of information on the encountering rates (number of encounters/total predators introduced) or on temporal strike rates (number of encounters resulted into attack in a given period of time). These two factors may be counted for the real number of prey removed from their habitat, which may be comparable to their control efficacy *in vitro*. An advanced experimental design should be devised to determine the characteristics of predatory nematodes in terms of their biological control potentials with the help of advanced techniques for analysing the experimental outcomes.

In comparison with other BCAs, the weakest aspect of predatory nematodes for the biological control of plant-parasitic nematodes lies in their low usability, including difficulties in their mass production and storage. These drawbacks can be alleviated to a certain degree to improve biological control efficacy by adopting the most feasible and practical approaches together with the development of techniques and methods of mass culturing and a long shelf-life in storage, especially referring to those in entomopathogenic nematodes. One of the control efficacy-related attributes, the predation ability of predatory nematodes, is almost equivalent to or higher than the antagonistic ability of other BCAs such as fungi and bacteria that rely mainly on competition, antibiosis and parasitism: the lethal activity of predation versus the inhibitory activities of competition and antibiosis in addition to the rapid and prompt effect of predation versus the slow and delayed effect of parasitism (Cook and Baker, 1983). Practical control efficacy would be higher in predatory nematodes due to their rapid dispersal from high motility compared to microorganisms with no or little motility. Summing up all of the advantageous attributes of predatory nematodes, their biological control potentials would be high and will be further enhanced by the development of more advanced mass production and storage techniques. The successful control of plant-parasitic nematodes using predatory nematodes may not only increase the qualitative and quantitative productivity of agricultural products (economic contributions) but also improve agricultural soil ecosystems since their predation activity leads to soil health improvement (ecological contributions). Following their successful establishment in the soil, their sustainable control activities will be exerted in the soil habitats that allow readily species co-existence, maintaining soil nematode diversity through niche partitioning, which may be responsible for the predominance of soil nematodes with high diversity in soil ecosystems (Ettema, 1998).

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Part VIII

Conclusions and Future Directions

18 Factors Affecting Commercial Success of Biocontrol Agents of Phytonematodes

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18.1 Introduction

Phytonematodes represent a global threat to agricultural production either as causing yield loss or quality loss, both included in the concept of crop loss. To date, approximately 4100 species of phytonematodes have been described that can cause various plant diseases (Decraemer and Hunt, 2006). They are of considerable importance and their deleterious impacts on crops have great economic and social effects. Total annual losses caused by phytonematodes in developing and developed countries are estimated to be about 14.6 and 8.8%, respectively (Nicol *et al*., 2011), that is approximately equal to US$157 billion (Abad *et al*., 2008; Escudero and Lopez-Llorca, 2012).

Management of phytonematodes is obligatory in addition to being a major challenge. Their current control method is primarily based on applications of highly toxic nematicides that are not only expensive but also sometimes remain unavailable for purchase (Sikora *et al*., 2003; Timper, 2011). Use of many chemicals is now banned or limited due to concerns regarding ecological risks and there is a strong need for alternative efficient control measures (Perry and Moens, 2006; Moosavi, 2012; Moosavi and Zare, 2012).

Biocontrol agents (BCAs) present an array of opportunities for crop protection, and numerous organisms are known with antagonistic activity against phytonematodes. However, until recently, only a small number have been commercialized for nematode control. This raises a question about the characteristics involved in the success of a BCA, though this subject also causes a number of nematologists to cast doubt on the qualification of biological control as an influential management measure. Some researchers believe that biological control is an unreliable, expensive and inadequate measure and its successful implementation is ambiguous, while others consider it an ideal and eco-friendly tool that should be used regardless of cost and low efficacy.

Based on many review articles which have collected the successful cases of biological control, the ability of fungi (Walia and Vats, 2000; Davies and Spiegel, 2011b; Hallmann and Sikora, 2011; Sharon *et al*., 2011; Moosavi and Zare, 2012) and bacteria (Walia *et al*., 2000;

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Tian *et al*., 2007; Gowen *et al*., 2008; Trivedi and Malhotra, 2013) in suppression of phytonematodes has been evidently confirmed in many monocropped plant hosts. These data noticeably validate the efficacy of BCAs in sustainable control of nematodes in monocropping cultivation, and propose biological control as a practical substitution for chemicals. Biological control has (debatably) also the potential to supply an acceptable enduring level of nematode management that is not provided even by engineered resistance in plants (Whipps and Davies, 2000). Therefore, the real challenge is not to prove the efficiency of biological control but to find and improve the factors that influence the potency of BCAs. BCAs must have a wide range of desirable traits that can be successfully introduced as a commercial product. There are several factors that influence commercial success of a potential BCA. In this chapter an attempt has been made to explain the key factors that determine the success and failure of BCAs (particularly bacteria and fungi) of phytonematodes during their commercialization.

In the presence of chemical nematicides with instant effect, which has received credit with being known as the saviour of Hawaiian pineapple and East Africa tobacco industries in the 1950s (Webster, 1998), BCAs have little chance for replacement unless they can prove their favourable efficacy. However, as a consequence of our growing knowledge on side-effects and environmental hazards of chemical pesticides, the general prospect of these eco-friendly products is considered to be positive. In the last decade, biopesticide sales have steadily grown by approximately 10% annually (Ravensberg, 2011a) and this was expected to increase by up to 15.6% in 2014 (Regnault-Roger, 2012). The market share of biopesticides in the global pesticide market was 3.5% (US$1.6 billion) in 2009 (Lehr, 2010). Though the most part of this small share was taken up by bioinsecticides, other products with activity against fungi, weeds and nematodes were also available (Wilson and Jackson, 2013).

It is evident from different research works that BCAs perform satisfactorily (Davies and Spiegel, 2011a; Timper, 2011; Moosavi and Zare, 2012; Trivedi and Malhotra, 2013), but inconsistency of their efficacy is the main reason that limits their use. Inconsistent performance may be related to the virulence loss (Lohmann *et al*., 1989; Zuckerman *et al*., 1989; Wang *et al*., 2003) or inadequate quality control in pre-application steps (Jenkins and Grzywacz, 2000; Ravensberg, 2011b). It may also be related to the involvement of more unknown and uncontrollable factors that a BCA encounters as the test scale increases (Dong and Zhang, 2006). All nematode controlling measures and their implementation require money, because their failure to suppress nematode populations can cause much more financial losses than when no money is expended on control. Greater input expense increases the expectation of the growers for more successful nematode control and higher yield. Therefore even little or incomplete nematode control is better than unreliable control (Timper, 2011). However, even a reliable BCA should only be accepted by growers if it is reproducible, cost-effective and user-friendly (Whipps and Davies, 2000; Whipps and Lumsden, 2001).

A successful BCA should survive application, keep its activity for a long time and could efficiently contend against other microbiota. Suitable inoculum form, feasible mass-production techniques, persistent and stable formulation, simple application tools and controllable quality should probably be supportive in successful introduction of a BCA (Whipps and Lumsden, 2001; Ravensberg, 2011a; Timper, 2011). The influential factors on commercial success of a BCA can be roughly divided into ecological, intrinsic, technological, societal, regulatory and commercial factors.

18.2 Ecological Factors

It is now well known that the lack of adequate knowledge about the impact of ecological factors on the performance of BCAs could be a reason for their failure (Deacon 1991, 1994; Kerry, 1995; Whipps, 1997; Whipps and Lumsden, 2001; Kerry and Hominick, 2002; Timper, 2011). Consideration of all important ecological principles at every step of selection, development and application of a BCA may significantly increase the longevity of its performance (Whipps and Davies, 2000; Timper, 2011). A better understanding of the interactions

of the BCAs with the target phytonematodes, the plant, environment (weather conditions and the edaphic factors) and the soil microbiota can lead to a successful BCA product (Fig. 18.1).

The ecological factors that have a considerable impact on the success or failure of the introduced BCA can be categorized into two factors, abiotic and biotic.

18.2.1 Abiotic factors

Soil can provide a good shelter for BCAs and protect them from environmental extremes of temperature and moisture, but it is also a complex and incongruous surrounding (Stewart *et al*., 2010). Soil consists of four major components: (i) mineral (or inorganic); (ii) organic; (iii) water; and (iv) air. The mineral matter could then be subdivided into coarse fraction (gravel, cobble, stone, boulder) and fine-earth fraction (sand, silt, and clay particles). Soil organic matter can originate from plant, animal, or microbial sources and may be relatively fresh or highly decomposed and transformed. The comparative proportions of these four soil components vary with soil type and climatic conditions (Brady and Weil, 2010). As performance of a microbial control agent is widely influenced by the environment into which that organism is introduced, endurance to fluctuation of abiotic factors is a fundamental trait for a BCA to act successfully.

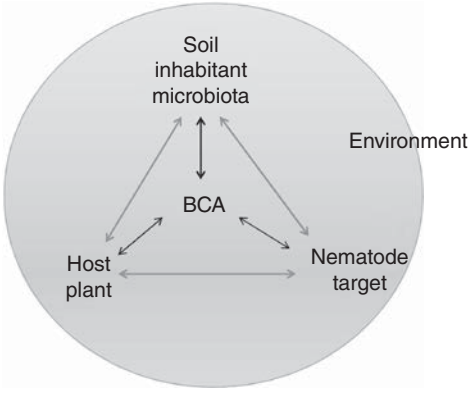

Fig. 18.1. Interrelationship among ecological factors that influence persistence and performance of a biocontrol agent (BCA).

UV irradiation is the most significant abiotic factor which is considered as a common cause of spore inactivation in the field (Dong and Zhang, 2006). It has been observed that exposure of *Arthrobotrys oligospora* and *Dactylaria candida* to UV irradiation for 5 min reduced their infection ability (Friman, 1993). Other abiotic factors that can affect the level of biological control include soil temperature, moisture, pH, organic matter content and nutritional status (Stewart, 2001; Timper, 2011). The interactions among abiotic factors are also very important, however in most cases it is not yet sufficiently specified (Stewart *et al*., 2010).

Soil temperature

Temperature obviously influences the persistence and performance of BCAs. The majority of fungal agents are mesophyllic, so low or high temperatures might restrain their biocontrol activity. Maximum fungal growth usually take place between 15 and 30°C, however different isolates may not have the same optima (Jaronski, 2007, 2010). Biocontrol activity of fungal BCA can be slowed down or completely stopped outside this range of temperature (Stewart *et al*., 2010). The case is also the same for bacteria and other BCAs. Efficient growth in a wide range of temperatures while retaining biocontrol activity is an essential trait for a successful BCA. A positive correlation was observed between increased temperature (increased from 15°C to 30°C) and adhesion of endospores of *Pasteuria penetrans* on the cuticle of second stage juveniles (J_2s) of *Meloidogyne javanica* (Stirling *et al*., 1990) and *M. arenaria* (Hatz and Dickson, 1992; Freitas *et al*., 1997). Attachment of endospores on *M. javanica* was reduced by 50% at 18°C as compared to 27°C (Stirling *et al*., 1990). Maximum infection of *M. arenaria* J_2 was achieved when *Pasteuria*-infested soil was kept at 20–30°C for 4 days. The spore attachment was found highest at 25°C (Talavera and Mizukubo, 2003), whereas at temperatures greater than 30°C adherence of spores was diminished (Freitas *et al*., 1997). The ability of a commercially available formulation of *Purpureocillium lilacinus* (strain 251) in decreasing the number of egg masses and galls of *M. hapla* was much

reduced when daytime temperature decreased from 23–25°C to 21°C (Kiewnick and Sikora, 2006).

Soil type

Soil type refers to the relative proportions of sand, silt and clay in a soil, which affects several soil properties like water intake or infiltration, water storage in the soil, soil aeration, soil fertility and trafficability (Brady and Weil, 2010). It is obvious that the ability for growth in different soil types is a worthy advantage, however most BCAs prefer a specific soil type and texture (Timper, 2011). *P. penetrans* prefers sandy soils rather than finer-textured soils (Mateille *et al*., 1995; Talavera and Mizukubo, 2003) and maintenance of its endospores in rhizosphere and adherence to J_2s of root-knot nematode can be controlled by soil type (Spaull, 1984). Nematode reproduction reduces significantly by *P. penetrans* in sandy-loam soils compared with loamy or clay-loam soils (Singh and Dhawan, 1992). The bacteria infected more females of *M. incognita* in sandy soil rather than in sandy-clay soil. A more uniform distribution of endospores (Mateille *et al*., 1996) or increase in mobility of J_2s in sandy soils could enhance the possibility of encountering the endospores of *P. penetrans* (Carneiro *et al*., 2007), however compared with fine textured soils, more endospores leach in sandy soils. After 24 h in sand, 76% of endospores leached about 10 cm under a drip system, but the leaching percentage decreased when clay was added to sandy soil (Dabire and Mateille, 2004). *P. penetrans* prefer soil texture where clay content is between 10 and 30% (Timber, 2011).

The effect of soil texture on natural occurrence, persistence and effectiveness of BCAs is not always identical (Stewart *et al*., 2010). Rhizosphere colonization and nematode controlling ability of *Pochonia chlamydosporia* was greater in peaty-sand as compared to loamy-sand or sand (De Leij *et al*., 1993). However, the fungus showed no preference in its establishment in compost, sandy-loam and loamy-sand soils (Siddiqui *et al*., 2009).

Soil moisture

Fungal BCAs usually have low level of osmotolerance and do not reproduce in dry soils. Low water potential restrains spore survival, conidial germination and germ tube and mycelial growth, however it has been reported that biocontrol fungi are usually more effective at lower levels of soil moisture (Stewart *et al*., 2010). Higher levels of soil moisture can reduce the persistence of fungal BCAs, and soil temperature has a direct effect on the rate of decline (Jaronski, 2007, 2010). Low impressibility from soil moisture (deficiency or excess) can help a BCA to perform persistently.

Soil water potentials below the wilting point of plants could decrease the activity of egg parasites (Roberts *et al*., 1981). High soil moisture decreased the controlling ability of *Hirsutella rhossiliensis* against beet cyst nematode, *Heterodera schachtii* (Timper and Brodie, 1993). The same results were also achieved against *M. javanica*. It seems that reducing nematode infection at high water potentials is related to less sporulation of this endoparasitic fungus rather than less movement of the nematodes (Tedford *et al*., 1992).

Contrasting results have been obtained in studies investigating effects of soil moisture on efficacy of *P. penetrans*. No relation was found between soil moisture and endospore adherence (Dutky and Sayre, 1978), while spore attachment was increased by moistening the soil 3 days before soil inoculation with J_2s of *M. incognita* (Brown and Smart, 1984). More spore attachment took place at soil moisture of 25.2% or 41.6% as compared to 9.5% (Talavera and Mizukubo, 2003). It was observed that irrigation and soil water-holding capacity affected the efficiency of *P. penetrans* because high and intense irrigation reduced the number of infected J_2s by leaching the bacterial endospores down the soil profile (Dabire *et al*., 2005). Siddiqui and Ehteshamul-Haque (2001) reported that efficacy and growth-promoting potential of *Pseudomonas aeruginosa* was greater at 50% or 75% moisture-holding capacity than at 25%.

Soil pH

Several agricultural practices such as soil amendment, implementation of fertilizers and organic component can alter soil pH, which in turn will influence the structure and diversity of soil microbial content. Variation in microbial population may consequently affect

the BCAs and change their diversity and effectiveness. Therefore, efficacy of BCAs might not directly and significantly be influenced by soil pH, but the indirect effect is not negligible (Stewart *et al*., 2010). Acceptable growth in diverse pH condition (alkaline, neutral and acidic) will help in commercial success of a BCA as well as high competitiveness against other microbiota at different levels of acidity. Activity of *H. rhossiliensis* was greatest when pH was approximately 4.5 (normal pH for acidic soils) and was minimal when pH was 6.5 (normal pH for neutral soils). Jaffee and Zasoski (2001) concluded that low pH decreases microbial activity, which somehow interferes with *H. rhossiliensis* pellets. Production of extracellular enzymes can be regulated by ambient pH, for instance the expression of serine protease gene (*vcp1*) of *P. chlamydosporia* increased under alkaline conditions (Ward *et al*., 2012).

Soil nutritional status

All microorganisms must have a supply of macro- and micronutrients, but they demonstrate great versatility in the means they employ to satisfy these requirements (Hogg, 2005). Microbial growth and sporulation as well as secondary metabolite production depends on a sufficient amount of mineral nutrition in soil. Thus, deficiency in soil nutrient can limit the competitive ability of a BCA in its environment and finally may restrict its biocontrol potential (Stewart *et al*., 2010; Ward *et al*., 2012; Santana-Gomes *et al*., 2013). The effects of zinc on polypeptide synthesis of biocontrol bacteria have been found altering the microbial population densities and changing the physiology of host plants (Behal and Hunter, 1995; Siddiqui and Shaukat, 2002; Shaukat and Siddiqui, 2003). Addition of zinc to soil enhanced the ability of *Pseudomonas fluorescens* CHA0 and *P. aeruginosa* IE-6S^{+} and suppressed the population of *M. javanica* (Siddiqui and Shaukat, 2002). Antagonistic behaviour of rhizobacteria against *M. javanica* increased in consequence of zinc application (Shaukat and Siddiqui, 2003). Implementation of zinc also considerably reduced the penetration of *M. javanica* into the roots of tomato (Siddiqui *et al*., 2002). Availability of a nutrient in a specific form as well as the soil nutritional status can influence microbial activity. Availability of nutrient influences the behaviour of nematopathogenic fungi (Mo *et al*., 2005). Nitrogen in ammonium form (Rodriguez-Kabana *et al*., 1981) and phosphorus in phosphite form (Cohen and Coffey, 1986) is preferred by many soil microorganisms.

Concentration of heavy metals

Heavy metals and their long-term persistence in soils form a serious threat for the quality of agricultural soils. These metals can greatly affect population, diversity and activity of BCAs as well as soil microbiota (Rosenzweig and Pramer, 1980; Gray, 1988; Ashour and Mostafa, 1999; Dumestre *et al*., 1999; Mo *et al*., 2006). The unfavourable consequences of heavy metals on microorganisms and their processes have been frequently documented (McGrath *et al*., 1995; Wang *et al*., 2007). Microorganisms respond variably to heavy metal, and resistance to heavy metals significantly enhance the activities of BCAs in the contaminated soil.

Interactions among soil abiotic factors

All the parameters mentioned above are very important in the performance of a BCA, but their interaction may be as significant as their individual effect (Stewart *et al*., 2010). It has been demonstrated that electrochemical characteristics of the soil, especially pH, has an overriding effect because adherence of endospores of *P. penetrans* on nematode cuticle is greatly influenced by cation concentration and pH (Afolabi *et al*., 1995). A more clear understanding of the interactions among soil abiotic factors can assist to close the gap between experimental results and commercial use of BCAs.

18.2.2 Biotic factors

Notwithstanding plenty of evidence in proving the importance of abiotic parameters, the impact of biotic factors must also be considered as a noticeable effector as well, since the results of experiments in sterilized soil are prominently different from those in non-sterilized

ones. Biotic parameters influencing the persistence and dissemination of natural BCAs of nematodes are poorly understood (Kerry and Hominick, 2002) and this may be due to the difficulty in identifying specific biotic impacts (Stewart *et al*., 2010).

Soil microbiota is comparatively stable. Usually one or more members of soil-inhabitant microorganisms act antagonistically against introduced BCA and limit its establishment. In many cases, antagonistic activity of the inhabitant soil microorganisms is the cause of poor establishment of introduced BCA, which ultimately causes poor nematode control, however, the casual agents involved in antagonism are mainly unidentified. It is supposed that competition for organic matter is the main cause that limits establishment of BCA in soil (Mankau, 1962; Cook and Baker, 1983) but it is also a fact that competition is not the only antagonistic behaviour which an introduced biocontrol microorganism encounters (Timper, 2011).

Soil organisms

The criterion for a successful BCA after its application is that it should retain its activity in the environment till the period when efficient control is needed. During this period, which might be several months, the applied BCA must survive hostile activity of the indigenous and competitive soil microorganisms (Whipps and Lumsden, 2001; Stirling, 2011). The soil microbial biomass in temperate grassland is very heavy as it is estimated that the weight of its inhabitant fungi and bacteria is 2–5 and 1–2 t/ha, respectively (Nannipieri *et al*., 2003). Introduced BCAs have to compete with this large volume of microorganisms.

Sustainability of the ecosystem contributes to the specificity relationships among antagonists; however, if the specificity is very high, the protection level of plants may decrease across different sites and from year to year (Yang and del Rio, 2002). In a competitive soil, saprotrophic BCAs are usually considered as feeble saprophytes and their propagule levels will greatly decrease in the lack of their nematode hosts (Stewart *et al*., 2010; Timper, 2011). Comparing the saprophytic growth of five *P. chlamydosporia* isolates in two sites with various soil microbiota, showed 57–98% growth reduction compared to in sterilized soil (Monfort *et al*., 2006). However, contrary results were also reported wherein *P. chlamydosporia* growth was inhibited by 0–37% in non-sterile soil (Siddiqui *et al*., 2009). A reverse correlation between egg colonization ability and saprophytic growth is reported suggesting a possible bilateral impact between them (Timper, 2011), however, no correlation was found between them by Siddiqui *et al*. (2009). Differential gene expression was found in saprotrophic-to-parasitic transition of *P. chlamydosporia* (Rosso *et al*., 2011).

Inhabitant soil microorganisms secrete metabolites that unfavourably influence propagule germination, growth of BCAs and their establishment. These secretions can intrinsically be toxic, resulting in lessened infectivity or multiplication (Stewart *et al*., 2010). A swelling in the spores and germ tubes of nematophagous fungi was reported as a result of applying *Bacillus* sp. secretion, iturin A-like compounds (Li *et al*., 2007). Secretions of two soil microbiota suppressed the growth of *P. chlamydosporia* and *P. lilacinus* (Monfort *et al*., 2006). *Pseudomonas fluorescens* metabolite, 2,4-diacetylphloroglucinol (DAPG), has been found involved in declining cyst and root-knot nematodes (Cronin *et al*., 1997; Siddiqui and Shaukat, 2003c). It has been illustrated that secondary metabolites from common soil fungi such as *Fusarium solani*, *Rhizoctonia solani* and *Aspergillus quadrilineatus* restrained DAPG expression (Notz *et al*., 2002; Siddiqui and Shaukat, 2003a, 2005; Siddiqui *et al*., 2004) and consequently the suppression ability of *P. fluorescens* against root-knot nematodes on tomato was reduced (Siddiqui and Shaukat, 2003a, 2005; Siddiqui *et al*., 2004). However, some other fungi such as *Pythium ultimum*, *Aspergillus niger* and *Trichoderma harzianum* increased antibiotic production of *P. fluorescens* without any significant enhancement in its biocontrol ability (Notz *et al*., 2001; Siddiqui and Shaukat, 2004; Siddiqui *et al*., 2004). The relationship between BCAs and soil microbiota is not always antagonistic. Several soil and gall-tissue inhabiting bacteria have been reported to enhance the adherence of *P. penetrans* spores to J_2 of *Meloidogyne* spp. (Duponnois and Ba, 1998; Duponnois *et al*., 1999).

Enterobacter cloacae when combined with *P. penetrans* raised the number of endospores production in the latter and enhanced its biocontrol activity (Duponnois *et al*., 1999). Soil invertebrates may also affect the BCA level in soil. Collembola and earthworms consumed spores and hyphae of fungal biocontrol agents (Jaffee *et al*., 1996; Jaffee and Muldoon, 1997). Nematopathogenic fungi have also been reported to be eaten by free-living nematodes (Bae and Knudsen, 2001; Okada *et al*., 2005).

Host plants

It has been demonstrated that the diversity, incidence, persistence and dynamics of BCAs as well as their influence on the management of nematodes might have been deeply affected by crop plant species (Kerry and Hominick, 2002). Compared with non-rhizosphere soil, the microbial populations have several orders higher magnitude in the rhizosphere where the nutrient concentration is more due to plant root exudates (Schloter *et al*., 1997). It may enhance the probable competition among the population of soil microorganisms. The leaked compounds by different plants may greatly vary and consequently alter the composition of the rhizosphere. Finally, it can boost or restrain the BCAs' incidence and persistence (Saxena *et al*., 2006), however the biocontrol activity does not necessarily change (Stewart *et al*., 2010). No correlation was found between the abundance of *P. chlamydosporia* (Bourne and Kerry, 1999) and *P. fluorescens* strain CHA0 (Siddiqui and Shaukat, 2003b) on roots, although their rhizosphere colonization was different among various host plants.

Crop tolerance or resistance to nematode attack influences the control level needed to avoid economic losses. The rate of nematode development and reproduction increases in a susceptible plant that might accordingly influence the effectiveness of BCAs. Some BCAs may be involved in signalling processes which induce resistance to nematodes in a number of plants (Kerry and Hominick, 2002). Unfavourable host plants, besides decreasing egg number per egg mass, enhance parasitism on sedentary stages of *Meloidogyne* spp. because small galls leave the egg sacs on the root surface exposed to biocontrol agents (Stirling *et al*., 1979; De Leij and Kerry, 1991; Bourne *et al*., 1996).

Nematode target

The nematode target probably has a crucial outcome in determining the successful deployment of BCAs. To appropriately comprehend the effect of BCAs on nematode populations, a good understanding of target nematode biology, ecology and population dynamics is necessary. Plant-parasitic nematodes might be migratory or sedentary endo- or ectoparasites (Kerry and Hominick, 2002). The strategy of BCAs against migratory nematodes is to create an adhesive or trapping structure, or to secrete immobilizing toxin which targets moving stages of nematodes while sedentary nematodes are usually parasitized by a number of opportunistic unspecialized BCAs (Moosavi and Zare, 2012). Availability along with successful infection of a nematode host is the method for significant reproduction of a BCA in soil. Therefore, there is usually a close correlation between the loading level of a BCA over time and the presence of susceptible nematode populations (Moosavi and Zare, 2012). Nematicide application reduces accessibility of suitable hosts, which results in decreasing the populations of BCA (Timber, 2011).

The growth rate of a nematode population can be of importance in the performance of a BCA. Biological control of nematodes with rapid population growth rate (*r* strategists) is more troublesome than nematodes with slow population growth rate (*K* strategists) (South-Wood and Comins, 1976; Kerry and Hominick, 2002). *Ditylenchus dipsaci* uses '*r* strategy' for reproduction whereas the majority of cyst nematodes are '*K* strategists'. Though *D. dipsaci* is an '*r* strategist', its long-term survival (Clayden, 1985) and population development (Atkinson and Dürschner-Pelz, 1995) can be considerably decreased by fungal BCAs. It seems that parasitizing the aggregated eggs rather than sporadic ones is easier for BCAs, however little is known about the natural parasites of these dispersed eggs due to their extraction difficulties (Kerry and Hominick, 2002). J_2s were found to be negatively correlated with the spore attachment of

P. penetrans (Talavera and Mizukubo, 2003). Storing in water suspension can lead J_2s to enter a quiescent-anoxybiotic stage (Antoniou, 1989). Nematode age is also important for its infection by the spores of *P. penetrans* (Davies *et al*., 1991). The separation of spores was observed in 1-day-old rather than 5-day-old juveniles (De Silva *et al*., 1996).

The lack of sufficient knowledge on the impact of ecological factors on the performance of BCAs is the main reason for their failure. Consideration of all significant ecological principles at every step of selection, development and application of a BCA may significantly increase the longevity of its performance. More understanding of the BCA's interaction with the nematode target, the plant, environment and the soil inhabitant microbiota can lead to the successful introduction of a BCA. The performance of a microbial control agent is widely influenced by the environment to which that organism is introduced, thus endurance to fluctuation of abiotic factors (such as soil temperature, type, moisture, pH, nutritional status) is a fundamental trait for a BCA to act successfully. The interactions among abiotic factors are also very important, however in most cases it is not yet sufficiently specified. Notwithstanding plenty of evidence in proving the importance of abiotic parameters, the effect of biotic factors must also be taken into account as a noticeable effector, since the results of experiments in sterilized soil are prominently different from those in non-sterilized ones. Biotic parameters influencing the persistence and dissemination of natural BCAs of nematodes are poorly understood, possibly due to the difficulty in identifying specific biotic influences. Biotic factors that have a major effect on BCA survival and effectiveness are soil organisms, host plants and nematode target.

18.3 Intrinsic Factors

There are some innate factors that affect successful establishment and performance of a nematopathogenic BCA such as aggressiveness, high competitive saprophytic ability, creation of robust survival structures, production of nematotoxic materials, compatibility with other control methods, pathogenic in a wide range of environmental conditions, safe to the environment, tolerant to antibiotics and agrochemicals, capable of establishing and reproducing in soil, promoting plant growth, inducing plant host defence mechanisms, and secretion of antibiotics and extracellular enzymes. However, among these the most important intrinsic factors in commercial success of a BCA are aggressiveness and persistence, which provide the reproducible levels of control. In spite of many successful small-scale trials, commercialization processes of several BCAs in large-scale field trials fail due to the lack of reproducible control (Whipps and Davies, 2000).

Different isolates of a BCA may differ in aggressiveness. For example, different isolates of *Pochonia* species, even those collected from similar soils, differed significantly in their parasitic ability (Moosavi *et al*., 2010). It is also reported that certain parasites could have interkingdom host-jumping from fungi to nematodes, insects or other animals (Nikoh and Fukatsu, 2000; Moosavi *et al*., 2011).

Killing the nematode target is of much interest as compared to changing behaviour or sub-lethal effects that can be recovered. However, competitive saprophytic ability is also vital for the BCA's survival and multiplication under diverse climatic situations, especially in the lack of a nematode target in and around the rhizosphere (Jackson and O'Callaghan, 1997; Desai *et al*., 2002). These traits will provide a routine and dependable performance for biological control.

The soil environment resembles a microorganism reservoir, where 1 g of fertile soil supports approximately as many as 3000 species (Ovreas and Torsvik, 1998), including 10^5–10^8 bacteria, 10^6–10^7 actinomycetes and 10^5–10^6 fungal colony forming units (c.f.u.) (Metting, 1993). Consequently, the more competitive the BCAs are, the more likely they will succeed. Rhizosphere colonization is considerably affected by specific recognition phenomena among controlling agents, nematode target species and physiological state of the plant (Kloepper *et al*., 1991). Production of extracellular enzymes and antibiotics as well as tolerance to antibiotics helps in successful establishment of BCA in the soil. Some fungal BCAs including *A. oligospora*, *A. microscaphoides*, *A. shizishanna*, *Monacrosporium megalosporum*,

H. rhossiliensis, *P. chlamydosporia*, *P. rubescens*, *Lecanicillium psalliotae* and *P. lilacinus* are known to produce several enzymes like serine proteases, serine carboxypeptidase, chitinases and chitosanases that are used to degrade the nematode cuticle and the eggshell wall (Moosavi and Zare, 2012).

There are arguments for and against host specificity. It would usually be desirable to have a BCA that is only pathogenic against one or more plant-parasitic nematodes on a range of crops (Kerry and Hominick, 2002), but not against non-target beneficial organisms. However, if a BCA can parasitize only one of several damaging organisms, the total cost of control might increase. Broader range of host specificity is probably a sign of the wider mechanisms (for example parasitism, predation, competition, etc.) used by BCAs (Stewart *et al*., 2010; Brodeur, 2012). Compatibility with other pathogen-controlling methods is also an essential trait for successful commercialization of a BCA. During a growing season a number of nematicides, fungicides and bactericides are usually applied on arable soils that could suppress BCA growth and establishment. A tolerant BCA can survive chemical application and may successfully integrate with them where short-term and long-term protection is provided by chemicals and BCAs, respectively (Desai *et al*., 2002).

According to Stirling (1991), our knowledge on the impact of pesticides against nematopathogens is insufficient. Implementation of fungicides may possibly decrease the activity of fungal BCA, however the recovery of *P. chlamydosporia* populations is also reported (Tobin *et al*., 2008). The tolerance of *P. penetrans* in many pesticides (Mankau and Prasad, 1972; Stirling, 1984; Chen and Dickson, 1998) as well as its susceptibility to chloropicrin (Kariuki and Dickson, 2007) has been demonstrated. Population densities of omnivorous and predatory nematodes decrease after nematicide application. Decrease in predatory nematode populations may result in increase of parasitic species. Omnivorous and free-living nematodes may serve as a food source for some BCAs (especially obligate parasites) and their scarcity in soil could affect the establishment of the BCA. In addition to direct action against nematode targets, many BCAs could promote plant growth, induce plant host-defence mechanisms against pathogens and/or secrete nematotoxic chemicals which might be of importance in elevating the control ability (Desai *et al*., 2002). Some known nematotoxic metabolites are bulbiformin (Brannen, 1995), phenazin (Toohey *et al*., 1965) and pyoleutorin (Howell and Stipanovic, 1980). It has been reported that the secretions of endophytic bacteria and fungi have a significant effect in inducing resistance (Siddiqui and Shaukat, 2003c; Siddiqui *et al*., 2005; Dababat and Sikora, 2007; Tesfamariam *et al*., 2009).

Besides the above-mentioned characteristics, other desirable intrinsic traits for successful development of a BCA are growth on diverse substrates, creation of a variety of survival and dispersal structures (endospores, chlamydospores, sclerotia, spores, hyphal fragments), adaptability and tolerance to different surrounding and edaphic conditions (Desai *et al*., 2002), safe to environment and non-target organisms including humans (Ahmad *et al*., 2011), compatible with other cultural practices and conducive to plant growth and soil health (Dong and Zhang, 2006).

18.4 Technological Factors

There are some specific technological obstacles that impede wide commercialization of BCAs. These barriers are usually related to scale-up production, formulation, stabilization and delivery technology. Numerous potentially excellent BCAs cannot be transformed from the experimental to the commercialization phase due to their inability to be mass produced (Stewart, 2001; Dong and Zhang, 2006), high cost for their production, unavailability of suitable and stable formulation, unpractical dosage advice (Khetan, 2001; Warrior *et al*., 2002), special conditions for their storage, inaccessibility to proper immobilizing materials and inappropriate delivery systems.

18.4.1 Production factors

Mass production on an industrial scale is one of the prime requisites for successful commercialization of BCAs. Extensive research

has been carried out, but inability to scale-up the production of many BCAs resulted in failure in their commercialization (Tormala, 1995; Stewart *et al*., 2010). It must also be taken into consideration that producing methods might be technically possible without being economically feasible. High production cost proves a deterrent to growers (Leggett and Gleddie, 1995). More efficient techniques are required for enhancing BCA production rate in order to achieve bulk propagule production with longer shelf-life.

A biocontrol microorganism that wants to meet commercial success must be able to grow on inexpensive media in a short period of time. After mass production, an inert or nutrient-based immobilizing material is needed that could carry the utmost number of BCA propagules for large-scale applications. Mass production without a need to be reared in or on another organism makes a BCA more suitable for commercialization (Campbell, 1989; Warrior *et al*., 2002).

Since BCAs with short shelf-life cannot be commercialized (Yang and del Rio, 2002), a reasonable shelf-life (at least 1 year) without the need of any special storage conditions (Desai *et al*., 2002) are other demanded traits of formulations to make them attractive for growers or for industry. Proper formulation of a BCA can maintain the stability of the propagules as well as the pathogenic potential during its shelf-life period when they are kept at ambient temperature during distribution systems and storage conditions of farms. The other keys to success of a BCA formulation are ease of implementation, applicability with equipment in use by growers and mixing ability with conventional products. The BCAs must be effective when applied at practical application rates.

Stabilization of environmentally sensitive beneficial organisms in formulation and pre- and post-application is also a requirement in commercialization. Formulation must be tailored to match the environs where the BCA is to be applied (Stewart *et al*., 2010).

A proper delivery system that introduces immobilized BCAs at appropriate place and time can significantly decrease the quantity of BCA required. The proper time and place of delivery is determined according to BCA, the pathosystem and the cropping system (Fravel and Engelkes, 1994; Fravel, 2005). Several techniques have been devised to produce effective carrier systems in commercial formulation. There are many carriers such as peat, seeds, meals, kernels, husks, bran, bagasse, farmyard manure, cow dung cake, compost, oil cakes, wood bark, vermiculite, sand, clay and liquid carriers that can be exploited in commercial production of biopesticides.

18.4.2 Quality control

Like other commercial ventures, quality control (QC) is one of the important challenges for the extensive adaptation of BCAs in manufacturing methods (Desai *et al*., 2002). QC can be divided into production control, process control and product control that supplies feedback on the production and formulation procedures and the final product (Ravensberg, 2011b). In addition to the lack of stringent guidelines for keeping good quality standards, it is much harder to uniformly produce living organisms and their resultant products than traditional chemicals (Desai *et al*., 2002). Therefore, devising a quick and reliable production and process control which could exactly quantify the active ingredient is essential for successful commercialization with minimum failures (Khetan, 2001). The parameters that must be carefully checked for a BCA product are the number of effective propagules, BCA purity, technical properties and effectiveness. A reliable product must also keep its specifications until the end of the claimed shelf-life (Ravensberg, 2011b).

Media used in commercial production of BCAs are not selective for only one particular organism; therefore probable contamination must be avoided since the quality and the final optimal population counts of the BCA will considerably decrease in contaminated cultures. Contaminated formulations are unable to achieve the expected results and also demonstrate differences among batches (Desai *et al*., 2002).

18.4.3 New technologies

As our knowledge of environmental factors influencing microbial populations in soil grows,

the chance of making a commercially successful product increases. Generation of such data is hindered by a historical lack of proper tools; however, some recent technological progress in molecular biology, biochemistry, genetics/genomic and developmental biology can be used. Antibodies have been implemented to quantify nematodes and their natural enemies in soil samples (Davies and Spiegel, 2011b). Several quick, sensitive and dependable techniques such as competitive and real-time PCR (Okubara *et al*., 2005) are now available for quantifying population densities of BCAs. Specific primers have also been designed for many nematopathogenic microorganisms (Hirsch *et al*., 2001; Atkins *et al*., 2003, 2005; Yin *et al*., 2003; Schmidt *et al*., 2004; Zhang *et al*., 2006; Smith and Jaffee, 2009). Sequencing the genome of BCAs and other related '-omics' technologies can help in improving the efficiency and effectiveness of a BCA (Manzanilla-Lopez *et al*., 2013).

New findings could considerably expand the potential of nematopathogens. With the help of molecular biology it is now possible to identify which organisms are responsible for nematode control in a suppressive soil, to follow the fate of introduced microorganisms and to determine how the populations of inhabitant and introduced BCAs fluctuate periodically and with different crop production practices (Timper, 2011). Molecular methods also enable the scientists to examine regulation of genes, stability factors and immune response factors. It is now feasible to monitor gene expression (such as antibiotic production, trap formation and parasitism) by reporter genes. For instance, expression of the antibiotic DAPG by *P. fluorescens* is studied by the aid of reporter gene *lacZ* (Notz *et al*., 2001, 2002). The *PII* gene of *A. oligospora* could be integrated with a reporter gene to specify the situations that make the fungus parasitic (Ahman *et al*., 2002). Microarray study could be used for *Monacrosporium haptotylum* to characterize the up-regulated genes in the adhesive knobs (Ahren *et al*., 2005).

Genetic engineering is an efficient tool for producing more aggressive organisms with their manipulated pathogenic genes. Probably in future, the use of genetically engineered microorganisms will strongly increase with the progress in technology. These microorganisms can be modified for over-expression of genes involved in biological control or nematicidal activity. Transgenic microorganisms could be more effective as compared to the wild-type strains (Sharon *et al*., 2001; Ahman *et al*., 2002; Siddiqui and Shaukat, 2003c; Yang *et al*., 2011).

18.5 Societal Factors

There are increasing public concerns over the implementation of agrochemicals and their adverse effects on environment and human health. These concerns have produced a demand for a more environmentally sustainable approach to agriculture and food-related industries. Replacing chemical control with biological control would help in the protection of natural resources, and will result in a significant reduction of environmental contamination. This will consequently protect non-target organisms from exposure to synthetic and toxic pesticides as well as improve sustainability in agricultural systems and enhance biodiversity.

Estimation of the damage value of these externalities on the ecosystem and human society is a difficult and complex assignment (Menzler-Hokkanen, 2006). Environmental and socio-economic costs of using pesticides, such as bird losses, groundwater contamination, pesticide resistance, public health impacts and loss in biodiversity, have been estimated in the USA in a series of papers summarized by Pimentel and Greiner (1997). The annual damage costs in the USA reached US$8.3 billion (approximately US$30 per person per year). It was reported that if it would be feasible to determine the full ecological and societal costs of using pesticides, the total cost would be considerably more than US$8.3 billion per year. The calculated damage costs have obviously surpassed the total purchase value of all pesticides, which is about US$6.5 billion per year. Therefore real costs of using pesticides are greater than two-fold of what is paid by the farmers, and could be considered as society financial assistance to the chemical control of pests (Pimentel and Greiner, 1997). These society subsidies are paid globally and its amount increases each year due to accumulation of contamination.

All of these along with public awareness provide a strong societietal pressure on government authorities for decreasing the use of chemicals. On the other hand, environmental activist groups and consumer organizations are pressurizing retailers and growers to decrease the amount of residues in fresh food (Ravensberg, 2011d). This gives rise to new modifications in regulations, as well as in political, cultural and social perceptions.

The substitution of BCAs for chemicals may take place if legal enforcement and social awareness withdraw chemicals from market (Whipps and Lumsden, 2001). Despite considering the public views and political tendencies as strong promotional factors, they should not be very determinative. The direction and target of a company is usually specified by market-driven demands (Ravensberg, 2011d). Adequate customer demand and market size in a reasonable time period is necessary to ensure a financial return on the investment in research and development of a BCA product. Society could have an indirect but significant role in generating market demand. A large-scale market will be available if society insists on buying organic products. However, if the prices of biological-based products are not cost-competitive with the chemicals, the consumers must willingly accept to pay more money for safer food, and also for living in an unpolluted environment.

There is another perception in society about biological control, which considers it with permanent danger as it is highly undesirable to have microbes on food. Therefore, it is vital to perform good communications with society to avoid such a 'food-scare' (Ritson and Kuznesof, 2006). It is recommended that biocontrol companies should invest in the education and training of their sales forces, their distributors and the end-users along with society to improve adoption of biopesticides (Marrone, 2007).

18.6 Regulatory and Commercial Factors

After selection, screening, characterization and formulation of a qualified BCA, there are many legal, financial and commercial challenges that must be conquered. There is a variety of regulations for the handling and use of BCAs.

18.6.1 Registration

Only a registered microbial control agent can be officially introduced into the market. The registration process is certainly the main hurdle for commercialization of microbial products. Generation and assessment of a complete registration dossier of a product is very elaborate, complex, time-consuming and expensive (Whipps and Davies, 2000; Desai *et al*., 2002; Ehlers, 2010; Ravensberg, 2011c). Registration requires a considerable amount of money, which fluctuates according to organisms, its field of use and country of submission. This amount of cost cannot be afforded by many companies and products, especially for the small companies or the products aimed at small markets. Registration can be a bigger problem if the procedures are compulsorily restarted in each country (Whipps and Davies, 2000). The costs for safe experiments and registration of a biological-based product are estimated to range between US$0.5million and US$3 million over the course of 4–8 years (Evans, 2004; Hokkanen, 2007; Marrone, 2007; Ehlers, 2010; Ravensberg, 2011c).

These problems have resulted in many products being brought to the market that do not meet registration processes and are sold as soil conditioners, plant-growth promoters, biofertilizers, biological activators or similar microorganism-based products that need no registration. However, use of these materials will constantly be joined with an un-estimated level of hazard since the data on toxicology, ecological impact and persistence of effect are not collected (Whipps and Davies, 2000; Whipps and Lumsden, 2001; Ehlers, 2011). To solve this abnormality, regulatory authorities are trying to facilitate the registration process and decrease its expenses for biological-based products (Whipps and Lumsden, 2001). On the other hand, the venture of companies is always in danger as the active ingredient (microorganism) can easily be isolated from the product. It means that the developed BCAs could be isolated, specified or used by anyone. Thus, protection of intellectual property

rights (product or technological idea) is vital for the future of the biopesticide companies (Tormala, 1995). Patent protection may be of help here. Biological processes, methods and organisms can be patented, however this will have an extra cost, ranging from US$50,000 to US$100,000. The patent process may take years, however its strength is not yet clear and it seems that genetic engineering will increase the complexity (Tormala, 1995; Ravensberg, 2011c).

18.6.2 Commercial factors

Devising a perfect business plan (BP) with all its detail is a prerequisite for starting the project. The BP must accurately predict the income, based on costs and profits, however the estimation of the potential revenues for the coming 6–10 years will not be easy. The costs include not only the starting resources but also the expenses of staying in business. The sales volume needs to be projected precisely and carefully, which needs an in-depth knowledge of markets and customers. A clear road-map could obviously be of help in the successful introduction of a new product (Ravensberg, 2011a). Capable management and personnel are crucial to the survival and success of a business. A qualified manager must have proficiency in areas such as finance, buying, selling, production and management of the personnel. Introduction of successful products is usually the result of a team working with adequate information on research, production, registration and marketing (Ravensberg, 2011a).

Development of a new microbial product costs many millions of Euros, and lasts about 5–8 years to reach a break-even point. It takes even more years to arrive at a sales volume that supplies a favourable profit. Therefore adequate resources are required to cover all expenses until sales can finally return those costs and produce profit (CPL, 2006b). In other words, quick profit-making is not possible and accordingly many businesses have withdrawn from this venture (Menzler-Hokkanen, 2006). Underestimation of the needed time and resources is the common mistake of many failures. Therefore in this field of business one needs comprehension, motivation, patience and diligence for being successful (Ravensberg, 2011a).

Sales can start when a product has been registered. The market acceptance for a new product is typically slow and the commencement of selling and consequent increase in sales requires a considerable effort of the producer and his distributors. Convincing farmers with a performance test of BCA under typical field conditions possibly in comparison with a commonly used nematicide as control, will be critical for better market acceptance of a new product. The companies could arbitrarily invest in production of biological or chemical products, but the main concern of many producers is market potential and financial income. Thus, a BCA will be commercialized only if it could guarantee an economic return (Khetan, 2001).

18.6.3 Market factors

Other restrictions in commercialization of a BCA are related to market share and market size (Whipps and Davies, 2000). Early analysis of the market size and potential market is essential to determine whether to continue the developmental process of a candidate BCA or not. It is recommended to assess the market size realistically during the earliest phase of any development procedure because overestimation of the potential share of market is considered as one of the main causes in failure of many companies. It must also be taken into consideration that the real market size is much smaller than the potential market size (Ravensberg, 2011a). The viable large market is a must to vindicate the huge money spent on the commercialization process and supply profit for the future (Desai *et al.*, 2002).

Another limitation in the commercialization process of a BCA is assigning a rational end-user price for the product. The price must be priced low enough to compete with the traditional chemicals that they are trying to substitute, and high enough to make a reasonable profit to producers for a sustainable business. The margin must be capable of covering

the development costs, a share of the cost of development of new products and a portion of the costs of failed products. However, in a competitive market the amount of profit that a producer can collect is limited by many factors. Allocating an appropriate price is a very complex subject in the business since the manufacturer's income must compensate for: (i) the fixed, variable and capital (depreciation, interest) costs; (ii) the overhead costs; and (iii) the profit margin (Tormala, 1995).

A business of producing BCAs can be considered successful only if the product is successful; and a product can be successful only if the company succeeds in marketing it for a long period of time. More sales can help in covering the expenses and making a reasonable profit. However, sometimes a company with a successful product may still fail because of inability to achieve its financial objectives (Ravensberg, 2011a).

18.6.4 Distribution factors

Distribution strategies in the success of biologically active compounds cannot be underrated. Efficient distribution strategies are needed for better marketing of biopesticides. Big companies usually have their own distribution organizations while the small ones need to use other companies as their distributors. Utilizing the large agrochemical distribution systems as distributors of microbial products has not resulted in successful marketing of biopesticides (CPL, 2006b) as compared to small local corporations. Using one's own distribution organization yields the best result (CPL, 2006a). Pricing and margins as well as the size of market and crops are determinative factors in this choice. The fundamental of the success of biological control with natural enemies and microbials lies in direct contact with the growers (Bolckmans, 1999).

A major barrier to the adoption of microbial product is the growers' perception about them, and the task of distributors and pest control advisors is critical for successful selling. It is suggested that biocontrol corporations invest in the learning of buyers and distribution channel colleagues to enhance adoption of microbial products (Marrone, 2007). Transferring the customer's satisfaction or complaints to the producer is another prominent role of distributors. Complaints may help in improving a product and will enhance the chance of products for commercial success (Ravensberg, 2011a).

To sell chemical products is easier in accordance to their storage, shelf-life, simplicity of application and advice. Furthermore, the income increases if the market size is larger. Therefore, convincing the distributors to sell more complex products like microbial ones is not simple, unless profit margins are high enough to encourage them. This subject must be taken into consideration by producers in calculating the end-user price. Distributors can play an important role in successful product launch in the market especially if the product is properly and timely introduced. These are very influential in the advancement of the products and identification of effective distributors in the way to success (Ravensberg, 2011a).

18.7 Conclusions

Biological control can be an appropriate substitute for chemicals if the factors that influence the potency of BCAs are precisely identified and improved. BCAs must have a wide range of desirable traits that can be successfully introduced as a commercial product. There are several factors that determine the final success or failure of a product in its commercialization. Confirmed market demand is a prerequisite for all commercial research and development. Ecological principles should be considered in isolation. Screening and characterization of potential BCAs should be tested for their aggressiveness and persistence. Some new technologies can help in improving the efficiency and effectiveness of a BCA. Genetic engineering is an efficient tool by which the pathogenicity genes can be manipulated and aggressiveness of organisms can be enhanced.

The barrier in commercialization is usually related to scale-up production, formulation, stabilization and delivery technology. Registration process is probably the main hurdle

for commercialization of microbial products. Finally, there are several commercial challenges related to price, distribution and market acceptance of new products. Capable management and personnel as well as knowledgeable distributors may be helpful in conquering commercial hurdles. Producers must consider these effective factors in all processes of commercialization. The more desirable traits a potential BCA has, the higher is the chance for it to be developed into a viable commercial product.

Transfer of knowledge is an important step in successful commercialization of a BCA. The growers must be aware of the fact that chemical nematicides have an adverse effect on soil health and at the same time also be convinced with the fact that BCAs are a safe alternative to chemicals and are ecofriendly and soil rejuvenating in nature. They must also be acquainted with limitations of using BCAs to have realistic expectations. When the limitations are comprehended, the advantages of BCAs like effective long-term control, flexible time in implementation, conserving environment, crop and soil health can be achieved.

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19 Limitations, Research Needs and Future Prospects in the Biological Control of Phytonematodes

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19.1 Introduction

Plant-parasitic nematodes (PPNs) constitute one of the major limiting factors in crop production. They cause extensive losses in yield of the crop, which in monetary term has been estimated up to US$358 billion annually on a worldwide basis (see Abd-Elgawad and Askary, Chapter 1, this volume). Besides quantity, quality of the crop is also severely affected. Use of pesticides is a quick and effective method of nematode management but reports in the last two decades revealed that nematodes have developed resistance against most of the known pesticides, which led to the search for a new option that would be environmentally safe and economically viable (Fernandez *et al.*, 2001). A well known chemical, methyl bromide, which was widely used against nematodes, has now been withdrawn from the market due to its adverse effects on the ozone layer (UNEP, 2001). Under this situation, use of biocontrol agents (BCAs) seems the best alternative. BCAs are those natural living enemies that are utilized deliberately as an ecofriendly pest management strategy to reduce the target pest population. Several BCAs such as predaceous and parasitic fungi, endoparasitic bacteria, predaceous mites, predatory nematodes, plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) are known to limit the population of PPNs. Fungal biocontrol agents *Paecilomyces lilacinus*, *Pochonia chlamydosporia*, *Trichoderma harzianum*, *Aspergillus niger* and *Arthrobotrys oligospora* are considered some of the best, safe and highly practicable options, and in the last two decades they have been successfully and commercially exploited by scientists against PPNs; however, none of them proved promising from all the corners (see Askary, Chapter 3, this volume). Among the bacterial biocontrol agents *Pasteuria penetrans*, *Pseudomonas fluorescens* and *Bacillus* sp. have shown great potential in the management of nematodes (see Eissa and Abd-Elgawad, Chapter 9 and Vagelas, Chapter 13, this volume), but commercial products of these bacteria possessing nematicidal potential are very few for application in the agricultural system (Whipps and Davies, 2000; Gardener, 2004; Schisler *et al.*, 2004). Predaceous mites such as *Lasioseius subterraneus* have been reported to devour >100 J_2 of root-knot nematode, *Meloidogyne incognita* per

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day, whereas *Pergalumna* sp. is reported to devour 18 J_2 of *M. javanica* and 41 lesion nematodes, *Pratylenchus penetrans* per day (see Gerson, Chapter 14, this volume), but due to lack of specificity against the nematode pest, difficulty in mass production as well as impractical field application, they are considered unsuitable for inclusion in a nematode biomanagement programme. The role of PGPR, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, *Azospirillum*, *Azotobacter* as biocontrol agents of PPNs have been well proved by research workers (see Wani, Chapter 15, this volume), but shortcomings of PGPR are that they remain active for a relatively short time and therefore are unable to provide long-lasting nematode control. In the past three decades certain species and strains of AMF used in laboratory and field were found to have biocontrol potential, mainly towards sedentary (*Meloidogyne*, *Heterodera*, *Globodera*) and to a lesser extent towards migratory endoparasitic nematodes (*Pratylenchus*, *Radopholus*) (see Sankaranarayanan, Chapter 16, this volume), however their specific mode of antagonistic action against nematodes is still not completely understood. On the other hand, predatory nematodes belonging to the order Mononchida, Dorylaimida and Aphelenchida have also been found successful in controlling PPNs, but their longevity and stability related to the shelf-life (storage), which are the main attributes for commercialization of a biocontrol agent, have been studied little by research workers (see Kim, Chapter 17, this volume).

In this chapter the aim is to highlight the critical issues involved in the successful utilization of BCAs against PPNs besides identifying the reasons that lead to general failure of the products that have been developed. I have paid specific attention on nematophagous fungi and bacteria with emphasis on future research needs to include them in an integrated nematode management (INM) programme.

19.2 Limitations of Biocontrol Agents

Numerous microorganisms thrive in the soil ecosystem with a complex network of interactions. These organisms compete for food sources particularly in the plant rhizosphere where the nutrients are in abundance. Applications of BCAs help in either protecting the host plants from infection of these deleterious microorganisms or reducing the severity of the disease. However, BCAs are more inconsistent and slower in action as compared to any other chemical methods. These limitations are inherent and therefore improvements in performance require more research on this aspect for successful application of BCAs in sustainable agriculture. There are certain bacteria and fungi that have the ability to suppress PPN populations directly through parasitism or indirectly by toxic metabolites (Dong and Zhang, 2006). A number of commercial products based on such nematophagous fungi and bacteria have been developed but with limited success only. The following sections consider the key factors hindering the utilization of BCAs against PPNs.

19.2.1 Isolation

Detection and quantification of a BCA in soil is the first step involved in its exploitation against target organisms. In the case of nematophagous bacteria, *Pasteuria penetrans*, which is an obligate parasite of active and sedentary nematodes and has a long endospores shelf-life, the main drawback is that they are poorly spread in soil and difficult to mass-culture under laboratory conditions. Species of nematode-trapping fungi such as *Arthrobotrys* have a wide host range besides having the advantage of being produced under *in vitro* conditions, but many species of these fungi do not form resting structures and therefore are difficult to formulate (Viaene *et al.*, 2006). The obligate nature of their parasitism also often prevents large-scale fermentation. Egg- and female-parasitic fungus, *P. lilacinus* has been exploited very successfully in several countries of the world, but limitations with this BCA are short duration survival and requirement of multiple applications in soil. Another successful fungus, *P. chlamydosporia* produces resting structures, i.e. chlamydospores (a survival structure resilient to environmental extremes), which are used as an inoculum for the establishment of

fungus in soil and rhizosphere; however, great variation among the isolates has been noticed in the ability to produce chlamydospores, root colonization and nematode pathogenicity.

19.2.2 Product cost

The product cost of BCA has an important role to play in its successful utilization and commercialization. Application of BCA is cost effective in the long run but in the initial stages it requires setting up of a biological control system which is undoubtedly a costly endeavour that requires extensive planning and high expenditure. BCA with high product cost may be acceptable for a crop of high economic value but the same is not true in the case of other field crops. Therefore, product cost of BCA must be relatively cheap as compared to nematicides, but that is often impractical.

19.2.3 Ecological factors

One of the main reasons for the failure of a BCA product is the lack of sufficient knowledge pertaining to the ecological impact on the activity of BCA. The relationships among the biotic parameters such as soil microorganisms, host plants and target nematode species are poorly understood by the users. The activity of AMF and PGPR are generally affected by a crop cultivar. PGPR are effective for a shorter time only and have no direct effect on the multiplication of nematodes. Therefore, while planning for a biological control, there must be a detailed understanding of BCA to be used, its biology and ecology and the nematode species to be targeted. A second limitation is that results obtained by BCA are often slow, which requires enough patience by farm workers to wait for BCA to work their magic against target nematode pests. The same is not true in the case of a pesticide, where the results are achieved immediately. An important point also to be noted is that if the nematode pest population is much above the economic threshold level and a quick solution is needed, BCA is not the suitable answer. Hence, advance planning is required before going for any biological control against PPNs.

19.2.4 Application technology

The method of application of BCA has an important role to play in the farming system (Davies *et al.*, 1991). By merely increasing the application of a BCA, it can only increase the amount of that particular BCA in soil but not necessarily in the rhizosphere. Rhizosphere bacteria when applied as seed treatment tend to provide short-term control against PPNs and that is confined to check or reduce the root invasion by nematodes in a single generation in one growing season only. If a BCA is effective against a specific nematode pest and the efficacy is dependent on pest densities, the effective use of such BCA will require expert advice working in the field and that seems to be a difficult task in many countries. Prior to application of a BCA, its mass production and proper formulation is necessary. Formulation refers to the preparation of a product from an ingredient by the addition of certain active (functional) and non-active (inert) substances. It provides the means to improve the activity, delivery, ease of use, storage stability and field efficacy of a BCA (Askary, 2010). Except for some fungi and bacteria, not much information is available on the mass production and formulation of biocontrol agents of nematodes and this may be due to lack of research on this aspect. Spores formulation in alginate pellets have resulted efficiently in the way of storage, distribution and application of fungi during biological control activity, but pellets and their contents may be destroyed by collembola and mites present in the soil and at high concentrations alginates may be phytotoxic (Viaene *et al.*, 2006). In case of *Pasteuria penetrans*, a minimum population level of its endospore determines the level of soil suppression, but it is to too difficult to calculate adequate endospore concentration in soil. There is also no mathematical equation correctly describing the relationship between the number of soil endospores and the level of soil suppression (Hallman *et al.*, 2009).

19.2.5 Variability in efficacy

The soil ecosystem is physically, chemically and biologically very complex. It harbours a variety of organisms with a complex network of interactions. The interactions between BCA, plant and nematodes in the rhizosphere is also complex. Temperature, moisture and density play a vital role in the activity of BCA, particularly fungi and bacteria. Nematode control with BCA is a slow process and it depends upon the efficacy of the BCA, the nematode species in question, the plant host and their root exudates, and other crops in rotation (Hallman *et al*., 2009). It has been observed that BCAs act well when tested under laboratory conditions but results are not so good when applied under field conditions and this may be due to the interactions of BCAs with the biotic and abiotic components of the surrounding environment. Other drawbacks of some BCAs are that they do not actively multiply in absence of host (e.g. *Pasteuria penetrans*) or remain active for a short period only (e.g. Rhizobacteria). Some isolates of *Pasteuria penetrans* are very host specific but their isolate selection is too difficult because spore burden is not always correlated with virulence (Viaene *et al*., 2006). In the case of trapping fungi, the trapping/capturing activity rarely coincides with the activity of infective stages of sedentary endoparasitic nematodes such as *Meloidogyne* and *Heterodera*. An effective use of egg- and female-parasitic fungi, *P. lilacinus* depends upon several factors, such as age, virulence, viability, inoculum concentration, method of application and environmental conditions. That is why *P. lilacinus* differs in virulence under varied conditions and their propagules are needed in large numbers for effective control of PPNs. *Pochonia chlamydosporia* has the limitation that its virulence potentiality varies with the nematode species, its density and plant host. The efficacy also varies with the host preference of the fungus. The isolates obtained from root-knot nematode may not exhibit equal virulence capability to infect the eggs of cyst nematodes than those isolated from cyst nematodes.

19.3 Research Needs

19.3.1 Optimum price

Availability of inexpensive BCAs for use against phytonematodes is the need of sustainable agriculture. For optimizing the cost of a BCA product, specific attention should be laid upon two points: (i) increasing the production efficiency; and (ii) reducing the process time. These can be achieved by bringing an improvement in media constituents and optimizing the process conditions. When the production will be at a larger scale, i.e. both at industrial scale and small scale, the price will decrease. The cheap and large-scale production of BCA accompanied with a long shelf-life will ultimately also bring an impact on market size.

19.3.2 Easy availability

The availability of biocontrol products can be made easier by increasing the production and its storage ability and these can be achieved by bringing an improvement in the formulations of BCA. Some successes have been achieved in the last few years as a number of fungal and bacterial BCAs have been formulated and commercialized in different countries of the world (Table 19.1).

Some of the most promising BCAs of PPNs which have been formulated for commercialization in the recent years are *P. lilacinus*, *Trichoderma* (fungus), *Pasteuria penetrans* (bacteria) and *Bacillus firmus* (rhizobacteria) (Dababat *et al*., 2006; Affokpon *et al*., 2011; Wilson and Jackson, 2013). Commercial products with *P. lilacinus* are marketed in Europe (in Italy), North Africa, Central America (Wilson and Jackson, 2013), the Philippines (Davide, 1990), China (Liu *et al*., 1996) and South Africa (EPA, 2005; Viaene *et al*., 2006), *P. chlamydosporia* has been commercialized in Cuba (Montes de Oca *et al*., 2009; Fernández-Larrea, 2012) and China (Mo *et al*., 2005), *T. harzianum* in South Africa and *A. niger* in India (Sen, 2000). Among the nematophagous bacteria, *Pasteuria penetrans* is being marketed in Japan, *B. firmus* in

Table 19.1. Commercial products of bacteria and fungi for the management of plant parasitic nematodes.

Microorganism	Product	Company/institution	Country
Fungus			
Paecilomyces lilacinus	BIOACT®WG	Bayer Crop Science	USA
P. lilacinus	Miexianning	Agricultural Institute, Yunan Academy of Tobacco Science	China
P. lilacinus	PL 251	Biological Control Products	South Africa
Pochonia chlamydosporia	KlamiC®	Rothamsted Research	UK
		Centro Nacional de Sanidad Agropecuaria	Cuba
Trichoderma harzianum	Romulus	Dagutat Biolab	South Africa
Bacterium			
Bacillus firmus	BioSafe-WP	Agro Green	Israel
B. firmus	BioNem-WP	Agro Green	Israel
B. firmus	Chancellor	Agro Green	Israel
B. firmus strain GB-126	VOTiVO®WP	Bayer Crop Science	Germany
Bacillus spp.	Nemix	Chr. Hansen	Brazil
Bacillus subtilis *B. licheniformis* *B. megaterium* *B. coagulans* *Pseudomonas fluorescens* *Streptomyces* spp. *Trichoderma* spp.	Pathway Consortia®	Pathway Holdings	USA
Pasteuria penetrans	Econem	Nematech	Japan
		Pasteuria Bioscience	USA
Pseudomonas fluorescens	Sudozone	Agriland Biotech	India

the USA and *Bacillus* spp. in Brazil (Hallman *et al*., 2009). At present, the key biological products are VOTiVO (*B. firmus*) and BioAct (*P. lilacinus*) (Wilson and Jackson, 2013).

19.3.3 Easy application

There are two approaches for application of BCAs in soil: (i) rapid control of a pest; and (ii) mass release of a BCA to provide long-lasting control (Brand *et al*., 2010). Prior to application of BCAs, the users should have a better understanding at a biological and ecological level about the interactions among BCAs, target nematode species, other soil microorganisms and the plant. The spores of nematophagous bacteria, *Pasteuria penetrans* have the ability to survive in air-dried soil for several years but its success to control soil-dwelling nematodes depends upon the distribution of spores in soil, which in turn is influenced by several factors such as soil type, soil moisture and temperature. Therefore, to make a BCA product successful among the end-users, scaling up of inoculum is necessary besides selecting a correct time for its application.

Under subsistence agriculture systems, crops are grown in mixed stands in relatively small areas, often having large labour inputs. As a result, application rates of an unformulated agent become relatively large. This unformulated agent can be mixed into the soil by hand, as long as the organisms are produced cheaply and locally. Thus, BCAs may be initially exploited for nematodes in developing countries (Hussey, 1990). Under a developed agriculture system, crops are grown in monocultures over large areas using standard application machinery and, therefore, in this situation formulated product of BCA possessing good shelf-life and low application dosages may be recommended against PPNs. One suitable option is seed treatment with BCAs, as it requires low input and therefore is economically viable (Askary, 2012). But prior to application in larger field areas its efficacy

needs to be ascertained where the soil population of nematode pests is too high.

Real-time or quantitative PCR (qPCR) provides a tool that aids in assessing the population dynamics of a particular BCA in soil (Stirling, 2011). The use of GPF-like fluorescent proteins as living cell markers (Wiedenmann *et al.*, 2009) helps to study *in vivo* the mode of action of the organisms transformed with the genes. This technology has been applied in the case of nematophagous fungi (Zhang *et al.*, 2008).

19.3.4 Enhancing efficacy

The prime requisite for successful management of PPNs through BCA depends upon: (i) establishment of BCA in soil; (ii) its self-perpetuating ability; (iii) ability to grow faster; (iv) survival under adverse condition; and (v) long-lasting virulence. These attributes are governed by biotic and abiotic factors such as soil conditions, nematode species, their reproduction rate as well as population density in soil and the host plant. Fungal BCA are usually applied to soil as spores and therefore they must be active and virulent for longer periods in colonizing the plant rhizosphere and rhizoplane, because the probability of the presence of sedentary female nematodes and eggs are more likely there. Kerry *et al.* (1993) have correlated successful establishment of fungal BCA with the availability of energy sources as they support the organisms to compete with the saprophytic fungus in soil. Kerry (2000) suggested some important criteria for isolate screening and strain selection of *P. chlamydosporia.* These were based on the ability to colonize the rhizosphere of plants and cultivars, ability to produce chlamydospores *in vitro* and percentage of parasitism on nematode eggs. However, a better understanding of the biology and ecology and metabolism from whole organism to molecular scales (Kerry and Hirsch, 2011) will lead to an efficient and effective use of *P. chlamydosporia*.

The correct choice of a crop in a rotation plan can increase the number of propagules meant for rhizosphere colonization during the next crop, in a plant–fungus compatible interaction (Manzanilla-Lopez *et al.*, 2013). This is required for achieving a significant level of nematode control. Researchers have developed some molecular techniques such as real-time polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and biological methods such as dilution plating on a selective medium, to monitor the occurrence, abundance and activity of the fungus in the soil, rhizosphere and nematode egg masses (Atkins *et al.*, 2003).

Endospores of nematophagous bacteria, *Pasteuria penetrans* have good shelf-life but they are difficult to culture in the laboratory and their mass production is an uphill task for the research workers. However, some successes have been achieved in the past few years. A company, Pasteuria Biosciences LLC (recently acquired by Syngenta), has successfully developed the mass production of endospores in a bioreactor, enough to conduct small field trials (Hewlett *et al.*, 2004, 2006). The hindrance coming in the way of obligate living conditions of bacteria was solved by regulating the activity of the sporulating protein Spo0F (Kojetin *et al.*, 2005). Understanding the populations of nematophagous bacteria and their action mechanisms against nematodes at the molecular level will provide a basis for improving the pathogenic activity of potential biocontrol strains (Tian *et al.*, 2007). This will also prove helpful in developing rational nematode management strategies.

A combination of two or more biocontrol organisms may result in a more effective management of nematodes than either of the organisms alone, because combined application has several advantages such as multiple modes of action against a target nematode pest, ability to infect the target nematode in more than one stage of its life cycle and ability to perform over a wider range of soil conditions. Sometimes, the combined application of two or more organisms may not show compatibility between/among themselves (Roberts *et al.*, 2005) and therefore prior to combined application, planning should be done very carefully in the selection of BCAs.

19.4 Future Prospects

Biological control is initially expensive, because a lot of planning and money goes in

setting up a biological control system, however it can be cost effective in the long run as BCA can sustain themselves in the environment due to their self-perpetuating nature. This also results in reducing the labour cost that is required with other control measures.

Combination of strains of two or more antagonists can be capable of providing an effective management of nematodes, but the effectiveness of strain combination cannot always be predicted from the performance of an individual strain. Sometimes a strain combination may not show compatibility between or among them and under such cases the performance is not up to desired level. Hence, while making a combination of more than one antagonist, researchers should pay specific attention to minimize the negative interactions. In the last two decades several companies of the world have successfully formulated and commercialized some fungal and bacterial BCAs with proven efficacy, but there is still scope for a variety of other microbial antagonists that are still untested or are in their advanced stages of research. For the last two decades research has being carried out for a perfect candidate that can be utilized in the management of PPNs, however this has not resulted in many commercial successes due to advantages and disadvantages of a BCA when it comes to mass production, formulation, long shelf-life and efficacy in wide-scale testing (multi-locational field trials). According to Hallman *et al.* (2009), for an effective biological control of phytonematodes, there is a need to understand the epidemiology, survival and mode of action of BCA even at the gene level where induced resistance is involved. Research should be emphasized on: (i) to investigate the mechanisms involved in stimulating the naturally occurring antagonists' potential in soils; (ii) to develop molecular methods to increase the detection of antagonists with high control potential; (iii) to develop effective application technology for seed treatment or transplants; and (iv) to improve understanding of modes of action for targeted development of new antagonists.

In the case of nematode pests, the general management practice for a crop is adopted before sowing of seeds or during transplantation of seedlings. This is because nematode attack mostly takes place on the underground plant parts when the crops are in the seedling stage. If the population of a particular nematode pest in soil is much above the threshold level, a slow management practice in the form of a BCA is not the answer. At this juncture an alternative quick control measure is the requirement, which may be a chemical nematicide. Because action of any BCA is not instantaneous, it requires time to act upon the pest and by the time the BCA would start working, much damage to the crop would have been done. Therefore, before going for any biomanagement practice, the potential environmental and economic impacts of the nematode pests upon the crop in question should be taken into consideration.

Biological control is a slow process and therefore cannot be a substitute for chemical control, which is meant for quick results, but in spite of this, biological control is widely accepted among growers as it is environmentally safe and free from health hazards. Variability in efficacy of BCAs under different environmental conditions makes it difficult to develop a BCA product that would prove effective worldwide. Nematode management requires a long-term protection and that is likely to be impractical and uneconomic. Therefore, a better way is to go for an integrated management approach. For implementation of integrated nematode management, the biological method is combined with other methods of management. This would require an accurate knowledge of the ecology, biology and action mechanisms of the organisms involved. Advances in molecular biology have provided information related to molecular mechanisms of action, which include production of nematotoxins, the signalling pathways that induce the host-plant defence mechanism, and the process of infection. Such valuable information can provide assistance in increasing the expression of toxins or enzymes from the microorganisms that would ultimately aid in the formulation and production of a nematicidal agent on a commercial scale.

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