Techniques for Work with Plant and Soil Nematodes

Edited by

Roland N. Perry, David J. Hunt and Sergei A. Subbotin

CABI

Techniques for Work with Plant and Soil Nematodes

This book is dedicated to John Southey. His book, *Laboratory Methods for Work with Plant and Soil Nematodes*, has been, and still is, a vital part of nematology literature and was the inspiration for this volume.

Techniques for Work with Plant and Soil Nematodes

Edited by

Roland N. Perry

University of Hertfordshire, Hatfield, Hertfordshire, UK and Ghent University, Ghent, Belgium

David J. Hunt

CABI, Bakeham Lane, Egham, Surrey, UK

and

Sergei A. Subbotin

Plant Pest Diagnostic Center, California Department of Food and Agriculture, California, USA and Center of Parasitology of A.N. Severtsov Institute of Ecology and Evolution, Moscow, Russia

CABI is a trading name of CAB International

CABI
Nosworthy Way
Wallingford
Oxfordshire OX10 8DE
UK

Tel: +44 (0)1491 832111
Fax: +44 (0)1491 833508
E-mail: info@cabi.org
Website: www.cabi.org

CABI
WeWork
One Lincoln St
24th Floor
Boston, MA 02111
USA

Tel: +1 (617)682-9015
E-mail: cabi-nao@cabi.org

References to Internet websites (URLs) were accurate at the time of writing.

A catalogue record for this book is available from the British Library, London, UK.

Library of Congress Cataloging-in-Publication Data

Names: Perry, Roland N., editor. | Hunt, D. J. (David J.), editor. | Subbotin, Sergei A., editor.
Title: Techniques for work with plant and soil nematodes / edited by Roland N. Perry, University of Hertfordshire, Hatfield, Hertfordshire, UK and Ghent University, Ghent, Belgium, David J. Hunt, CABI, Bakeham Lane, Egham, Surrey, UK, Sergei A. Subbotin, Plant Pest Diagnostic Center, California Department of Food and Agriculture, California, USA and Center of Parasitology of A.N. Severtsov Institute of Ecology and Evolution, Moscow, Russia.
Description: Wallingford, Oxfordshire ; Boston, MA : CAB International, [2021] | Includes bibliographical references and index. | Summary: "This up-to-date, comprehensive book covers the practicalities of working with and studying soil and plant nematodes. Written by an international team of experts, it includes traditional and new methodologies, as well as sections on behavioural and physiological assays, and cytogenetic, biochemical and molecular biology techniques"-- Provided by publisher.
Identifiers: LCCN 2020026156 (print) | LCCN 2020026157 (ebook) | ISBN 9781786391759 (hardback) | ISBN 9781786391766 (ebook) | ISBN 9781786391773 (epub)
Subjects: LCSH: Plant nematodes. | Soil nematodes.
Classification: LCC QL391.N4 T388 2021 (print) | LCC QL391.N4 (ebook) | DDC 632/.6257--dc23
LC record available at https://lccn.loc.gov/2020026156
LC ebook record available at https://lccn.loc.gov/2020026157

ISBN-13: 9781786391759 (hardback)
9781786391766 (ePDF)
9781786391773 (ePub)

Commissioning Editor: Rebecca Stubbs
Editorial Assistant: Lauren Davies
Production Editor: Tim Kapp

Typeset by SPi, Pondicherry, India
Printed and bound in the UK by Severn, Gloucester

Contents

About the Editors

Roland N. Perry

Professor Roland Perry is based at the University of Hertfordshire, UK. He graduated with a BSc (Hons) in Zoology from Newcastle University, UK, where he also obtained a PhD in Zoology on physiological aspects of desiccation survival of *Ditylenchus* spp. After a year's post-doctoral research at Newcastle, he moved to Keele University, UK, where he taught Parasitology; after 3 years at Keele, he was appointed to Rothamsted Experimental Station (now Rothamsted Research). His research interests centred primarily on plant-parasitic nematodes, especially focusing on nematode hatching, sensory perception, behaviour and survival physiology, and several of his past PhD and post-doctoral students are currently involved in nematology research. He remained at Rothamsted until 2014, when he moved to the University of Hertfordshire. He co-edited *The Physiology and Biochemistry of Free-living and Plant-parasitic Nematodes* (1998), *Root-knot Nematodes* (2009), *Molecular and Physiological Basis of Nematode Survival* (2011), *Plant Nematology* (first edition 2006 and second edition 2013) and *Cyst Nematodes* (2018) (all CAB International, UK). He is author or co-author of over 40 book chapters and refereed reviews and over 120 refereed research papers. He is joint Editor-in-Chief of *Nematology* and Chief Editor of the *Russian Journal of Nematology*. He is joint Editor of the book series *Nematology Monographs and Perspectives*. In 2001, he was elected Fellow of the Society of Nematologists (USA) in recognition of his research achievements; in 2008 he was elected Fellow of the European Society of Nematologists for outstanding contribution to the science of Nematology; and in 2011 he was elected Honorary Member of the Russian Society of Nematologists. He is a Visiting Professor at Ghent University, Belgium, where he lectures on nematode biology, focusing on physiology and behaviour.

David J. Hunt

Dr David Hunt gained his BSc at the University of Nottingham, UK, and his PhD at the University of Reading, UK. From 1975 to 1979 he worked as a field nematologist in the West Indies and has travelled widely in the Caribbean region and in eastern and southern Africa. From 1979 until 2010 he worked in the UK as a nematode biosystematist for CABI, first at the International Institute of Parasitology and then at CABI Bioscience, where he is now an Emeritus Fellow. He has taught many international courses in nematode identification, these being mainly based in the UK, but also in Africa and Central America. Since 2002 he has been joint Editor-in-Chief of the journal *Nematology* and joint Series Editor of *Nematology Monographs and Perspectives*. He has published over 100 new nematode taxa, working mainly with parasites of diplopods and insects, contributed numerous chapters to a broad spectrum of books, authored the book *Aphelenchida, Longidoridae and Trichodoridae: Their Systematics and Bionomics* (1993) (CABI International, UK) and, with Khuong Nguyen, co-edited the monograph *Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts* (2007) and the subsequent companion volume *Advances in Entomopathogenic Nematode Taxonomy and Phylogeny* (2016) (both Brill, The Netherlands).

Sergei A. Subbotin

Dr Sergei Subbotin obtained an MSc in Plant Protection in 1981 from the Moscow Agricultural Academy in Russia. In the same year, he joined the Plant Pathology Laboratory in the V. R. Williams All-Russian Fodder Research Institute, Moscow region, and later All-Russian Scientific Research Institute of Fundamental and Applied Parasitology of Animals and Plants named after K. I. Skryabin (former VIGIS), Moscow, where he was awarded a PhD in Helminthology (Nematology) in 1987. Dr Subbotin has worked as a researcher in the

Center of Parasitology of the A. N. Severtsov Institute of Ecology and Evolution (Moscow, Russia), Rothamsted Experimental Station (now Rothamsted Research, Harpenden, UK), Institute for Agricultural and Fisheries Research (ILVO, Merelbeke, Belgium), Julius Kühn-Institut (Münster, Germany), Department of Nematology, University of California (Riverside, USA) and as a Visiting Professor in Ghent University, Belgium. Presently, he serves as Senior Nematologist in the Plant Pest Diagnostic Center, California Department of Food and Agriculture, Sacramento, California, USA, and has affiliation positions as Scientist in the Center of Parasitology, Russia and Associate Scientist in the University of California, USA. Dr Subbotin is one of the Founders and Chief Editors of the *Russian Journal of Nematology*. He has published more than 190 scientific articles in peer-reviewed journals on different aspects of molecular systematics and diagnostics of nematodes, and is the author of 22 book chapters. He is co-author of the two volume *Systematics of Cyst Nematodes (Nematoda: Heteroderinae)* (2010) (Brill, The Netherlands), *Systematics of the Sheath Nematodes of the Superfamily Hemicycliophoroidea* (2014) (Brill) and *Entomoparasitic Nematodes of the Order Tylenchida and Aphelenchida* (2019) (in Russian; KMK, Russia), and co-editor of the two volume *Plant Parasitic Nematodes in Sustainable Agriculture of North America* (2018) (Springer, Germany). His research studies have focused on molecular systematics, diagnostics and phylogeography of nematodes.

Contributors

Janete A. Brito Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, FL, USA (Janete.Brito@fdacs.gov)
Philippe Castagnone-Sereno INRAE, Université Côte d'Azur, CNRS, ISA, Sophia Antipolis, France (philippe.castagnone@inrae.fr)
Myriam Claeys Nematology Research Unit, Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium (Nini.Claeys@UGent.be)
Matthias Daub Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Field Crops and Grassland, Dürener Straße 71, 50189 Elsdorf, Germany (matthias.daub@julius-kuehn.de)
Loes J.M.F. den Nijs National Reference Centre, Netherland Food and Consumer Product Safety Authority, Geertjesweg 15, 6706 EA Wageningen, The Netherlands (loes@mulderdennijs.nl)
Ralf-Udo Ehlers e-nema GmbH, Gesellschaft für Biotechnologie und biologischen Pflanzenschutz, Klausdorfer Str. 28 - 36, 24223 Schwentinental, Germany (ehlers@e-nema.de)
Jonathan D. Eisenback School of Plant and Environmental Sciences, 103 Price Hall, Virginia Tech, Blacksburg, VA 24061, USA (jon@vt.edu)
Johannes Hallmann Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Messeweg 11/12, 38104, Braunschweig, Germany (johannes.hallmann@julius-kuehn.de)
Danny A. Humphreys-Pereira Laboratory of Nematology, Crop Protection Research Center (CIPROC), Agronomy School, University of Costa Rica, San José 2060, Costa Rica (danny.humphreys@ucr.ac.cr)
David J. Hunt CABI, Bakeham Lane, Egham, Surrey, TW20 9TY, UK (d.hunt@cabi.org)
Taeho Kim Division of EcoScience, Ewha Womans University, Seoul 03760, Republic of Korea (thk0817@gmail.com)
Jan L.M. Leunissen Nematology Research Unit, Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium (leunissen@xtra.co.nz)
Rosa H. Manzanilla-López Centro de Desarrollo de Productos Bióticos de México, Instituto Politécnico Nacional, Carretera Yautepec-Jojutla, Km. 6, Calle CEPROBI, No. 8, Col. San Isidro, Yautepec, Morelos. C.P. 62736, Mexico (rosa.manzanilla@gmail.com)
Leendert P.G. Molendijk Wageningen University & Research/Field Crops, Pb 430, 8200 AK Lelystad, Edelhertweg 1, 8219 PH Lelystad, The Netherlands (leendert.molendijk@wur.nl)
Joong-Ki Park Division of EcoScience, Ewha Womans University, Seoul 03760, Republic of Korea (jkpark@ewha.ac.kr)
Roland N. Perry School of Life and Medical Sciences, University of Hertfordshire, Herts AL10 9AB, UK (r.perry2@herts.ac.uk)
Jon Pickup Virology and Zoology, SASA, Edinburgh, EH12 9FJ, UK (jon.pickup@sasa.gov.scot)
Sergei A. Subbotin Plant Pest Diagnostic Center, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832, USA (sergei.a.subbotin@gmail.com)
Nicole Viaene Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Plant Sciences Unit, Crop Protection, Burg. Van Gansberghelaan 96, 9820 Merelbeke, Belgium (nicole.viaene@ilvo.vlaanderen.be)
Wim Wesemael Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Plant Sciences Unit, Crop Protection, Burg. Van Gansberghelaan 96, 9820 Merelbeke, Belgium (wim.wesemael@ilvo.vlaanderen.be)
Weimin Ye Nematode Assay Section, Agronomic Division, North Carolina Department of Agriculture & Consumer Services, 4300 Reedy Creek Road, Raleigh, NC 27607, USA (weimin.ye@ncagr.gov)

Vladimir V. Yushin A.V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok 690041, Russia (vvyushin@yandex.ru)
Julia K. Zograf A.V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok 690041, Russia (zojulia@yandex.ru)

Preface

Nematodes are an amazing group of animals, impacting human life in various ways, often with detrimental effects. Plant-parasitic and free-living nematodes are increasingly important in relation to food security, quarantine measures, ecology, including pollution studies, and research on host–parasite interactions. Most plant-parasitic and free-living nematodes are microscopic and are challenging organisms for research, as well as being difficult subjects to convince growers and advisory workers of their economic importance. A plethora of information on methodology for work with nematodes is available. In a single volume it is not possible to cover all available information. However, there is a need to unify approaches, especially in relation to descriptions, sampling and quarantine investigations, and to ensure that users are aware of various techniques that are available. Equally, research methodology needs to be summarized and bought together.

These chapters aim to provide an introduction to basic techniques for laboratory and field work with plant-parasitic and free-living soil-dwelling nematodes. The coverage highlights areas that have expanded and/or become more widespread over recent years, such as techniques used in diagnostic laboratories, including computerized methods to count and identify nematodes, and the use of entomopathogenic nematodes as environmentally acceptable control systems for some insect pests. The use of molecular techniques is relevant to many areas of work on nematodes and basic information on current molecular methodologies and their various applications is included.

This book has been collated with nematology students in mind, but the spectrum of information may also be useful for established workers. There is a conscious effort to include some classical studies and techniques that have proved invaluable over the years to nematologists and are still relevant. However, some of the chemicals that were previously widely used, and with little restriction, are either no longer available or are banned due to toxicity and/or environmental concerns. This necessitates use of alternative techniques or more effective control systems to ensure operator and environmental safety.

We are grateful to the chapter authors for their time and dedication in contributing to this volume. Their expertise has been essential in providing base-line information of available techniques.

Roland N. Perry
David J. Hunt
Sergei A. Subbotin
April 2020

1 Sampling

LOES J.M.F. DEN NIJS,[1] JON PICKUP[2] AND RALF-UDO EHLERS[3]

[1]*National Reference Centre, Wageningen, The Netherlands;* [2]*SASA, Edinburgh, UK;* [3]*e-nema GmbH, Schwentinental, Germany*

1.1 Introduction: The Purpose of Sampling

The main drive for sampling is to know what is in there, 'there' being the matrix that one is interested in. This can vary from water, soil, wood, turf or bark, to any part of plants (seeds, stems, leaves, bulbs, etc.), as long as it concerns the plant-parasitic nematodes. When animal-parasitic nematodes are involved, the matrix will be parts of the infected animal, and when we look into entomopathogenic nematodes, soil and the parasitized insects will be the point of attention for sampling.

Nematodes can be present in various parts of plants or at various depths in the soil, depending on the circumstances and the life stage. This means that one should be aware of these possibilities when collecting samples. When soil is too wet, it is better to wait until the soil has reduced to field capacity. The same is true for when the soil is too dry. Nematodes need water and in dry conditions the nematodes will move to deeper soil layers. In general, soil samples can best be taken when the soil is moist. Some life stages are immobile and can only be found in the plant roots, whereas other stages can be found in the soil. This means that samples should be taken from the proper matrix at the appropriate time, taking into account the developmental stages the nematodes might be in. All the above-mentioned aspects should be kept in mind when sampling for nematodes.

In this chapter, the purpose of sampling, sampling techniques and, related to this, the sampling tools, and the handling and storage of the samples before processing of plant-parasitic and entomopathogenic nematodes will be discussed. Some protocols will be described in detail as examples.

1.2 Sampling Strategies

1.2.1 Diagnostic sampling

Plant-parasitic nematodes are generally not visible to the naked eye and the symptoms they produce are often attributed to fungi, viruses, bacteria, other pests or poor soil conditions, including a deficiency or excess of nutrients. As nematode damage can easily be overlooked, it is very important to sample plants and/or soil for

 Techniques for Work with Plant and Soil Nematodes
(eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
DOI: 10.1079/9781786391759.0001

nematodes when growth is unexpectedly poor or when symptoms cannot clearly be attributed to other causes. This type of sampling is called diagnostic sampling, as the reasons for poor growth of plants or trees need to be determined. In these situations a sample from the poorly growing plant/tree should be compared with a sample from a healthy one. When possible, a soil, leaf or root sample from the middle of the poorly growing patch of plants should be taken. For poorly growing trees samples from roots, leaves/needles or borings from trunks may be necessary. For comparison, a sample should also be taken from a healthy-looking plant/tree and/or the soil beneath it. In addition, when the patch of poor growth is clearly visible, it is wise to take a soil sample from the transition area (the area between healthy and poorly growing plants). Where plants are dead, no sample should be taken from that area or the dead plants as the nematode population is likely to have decreased markedly under these circumstances. The sample should then be taken from the less vigorous plants. In all situations, the nematodes have to be extracted from the plants/trees and soil using the appropriate technique: nematodes can be found in different parts of plants such as the roots, leaves and growing tips, and each matrix needs its own extraction method to separate the nematodes from the tissue or soil (see Viaene *et al*., Chapter 2, this volume). If possible, roots should either be included in the sample or taken separately; about 25–100 g, taken at random, should be sufficient, the lower weight being suitable for vegetables or citrus, whilst the higher weight being more applicable to plants with large roots such as banana. If stems and/or leaves appear to be attacked by nematodes, affected material can be removed and placed in polythene bags. Such samples should be kept separate from soil and/or root samples. To avoid misinterpretation, comparison of the nematodes found in the different situations can help determine whether nematodes in general or specific species/genera are involved.

In summary:

- Determine the poorly growing patch of plants/trees based on symptoms.
- Sample the soil from the centre of the poorly growing patch (but not under dead plants) using an auger or coring device, small trowel or a narrow-bladed shovel. A minimum of 500 g of soil should be taken.
- Take a complete plant sample from the centre of the patch of poor growth (but not a dead plant/tree).
- Repeat the above for the seemingly healthy situation and for the transition area when this is visible.
- Make sure that each sample is put into a (polyethylene) bag with label attached or inside and fill in all the necessary information to trace the sample back to its origin, e.g. sampling date, location (GPS coordinates), crop and cultivar plant species, name of sample taker, name of owner of crop/farmer, a reference number (when more samples are taken on one site). If possible, include details of the previous crop, soil type, treatments and other relevant information.
- If it is suspected that the pine wood nematode, *Bursaphelenchus xylophilus*, might play a role, wood from the trunk should be collected. See Section 1.7 for more detail and the sampling protocol.

1.2.2 Sampling for detection

When sampling for detection the question is usually 'Are nematodes present in the field?'. When the objective is to detect the nematode in situations where the nematodes should not be present (see Section 1.3), sampling is similar to that for density estimates but the intensity of the sampling will be greater, related to the required detection level and the known distribution of the nematodes in the sampling unit. Sampling units might be soil samples, bulbs, plants or part of plants such as roots or growth tip.

Depending on the nematode groups likely to be responsible for the damage, the sampling depth may be very important. Nematodes are very mobile and avoid dry conditions. For virus vector nematodes, such as the genera *Longidorus*, *Xiphinema* and *Trichodorus*, Brown and Boag (1997) described the vertical distribution of these virus vectors as varying from 0 to 180 cm in depth. As these nematodes can be found deep in the soil, an auger of at least 40 cm depth should be used to collect soil samples. When the soil is too dry, in sub-tropical and tropical areas or in summer in temperate regions, the nematodes will have moved to deeper soil layers (even up to 100 cm depth) and soil sampling will become very difficult using a standard auger. In these situations a trowel can be used to remove the dry soil until the moist soil is reached and then take a soil sample. In general, a soil sample taken at ploughing depth should be sufficient in most cases in annual crops. In perennial and tree crops, soil sampling should take place as near to the root system as possible as Padusaini *et al.* (2006) already showed that the presence of the root system is the determinant factor in the vertical distribution of the nematodes.

Much current guidance suggests that when sampling for sedentary nematodes, such as cyst nematodes, cores should be taken to a depth of 15 cm, but there is little evidence to support this. Research by Been and Schomaker (2013) in The Netherlands examined the distribution of potato cyst nematode (PCN) cysts within a vertical plane and they concluded that there was a uniform distribution of cysts within the top 25 cm of the soil profile, both directly after harvest and after cultivation. Boag and Neilson (1994) concluded that sampling at any depth within the top 20 to 25 cm is suitable. Apart from changing the volume of soil collected, changing the size of the corer has minimal impact on changing the accuracy of the population estimation (Been and Schomaker, 2013).

1.2.3 Sampling for density estimates

When the question is 'Which nematodes and how many nematodes are present in the field?', especially for advisory purposes, it is important to estimate the density of the damaging nematodes. In these situations the soil sample should be representative of the area for which information is needed; this means that the sample should be taken in a representative way over the whole of the area and at the appropriate time. In most situations a soil sample should be taken, but in some situations bulbs, tubers or plants might be sampled as well.

For soil sampling, knowledge of the distribution pattern and biology of the nematodes is essential. The density of nematodes fluctuates depending on the life cycle and the host stage. For example, for advisory purposes in arable crops, sampling when maximum population densities are reached, often at the end of the growing season after harvest, may reduce the level of error associated with sampling. However, as crop damage is likely to be greatest at the highest population levels, sampling before planting a host crop is generally a necessity for making decisions on how best to protect the crop. For perennial hosts, sampling should be conducted during the active growing period, being the rainy season in tropical areas and springtime and summer in temperate areas (Coyne *et al.*, 2014).

The number of cores, the amount of soil, the grid pattern and the direction of sampling are all factors influencing the accuracy of the resulting estimate of the population density (Duncan and Phillips, 2009). Many approaches to sampling can be found in the literature (e.g. McSorley, 1982; Prot and Ferris, 1992; Been and Schomaker, 2013). A pragmatic approach to sampling is to find an optimal balance between the quantity of material collected, the number of sampling points and the cost of processing those samples. Factors to be taken into consideration include the financial benefits of knowing the nematode population density in terms of likely loss of yield and the reliability of the diagnostic method. Computer programs have been developed for this purpose such as SAMPLE, which was developed to evaluate existing, and create new, sampling methods for the detection of infestation foci of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* (see Been and Schomaker (2000) for more details). Another approach is the advice that Coyne *et al.* (2014) give in their practical field and laboratory guide for all soil sampling situations: take 10 to 50 cores/subsamples to form a composite sample from each hectare of soil. Table 1.1, taken from the operational guidelines of the Advisory Services for Nematode Pests (Stirling *et al.*, 2002), contains useful information for determining how to sample in various situations.

In the case of plant or plant products, the number of plants and the place they are taken from are important for the accuracy of the density estimate. The greater the number of sampling units taken for analysis, the more accurate will be the estimate.

In summary:

- Determine the area of the survey requirements or research object.
- Determine the nematode species of the required survey. In some cases all nematode species are of interest.
- Depending on the life cycle of the nematode, decide to take a soil sample, a plant and/or root sample or both.
- Take a composite soil sample by taking cores while walking in a systematic pattern over the area of interest. Take as many cores as possible, as more cores means more accuracy; however, this should be balanced by the available time, resources and requested accuracy.
- Take as many units as feasible to form a composite sample of the part(s) of plants the nematodes will be in; however, this should be balanced as above.

Table 1.1. Issues to be considered when collecting predictive samples for nematodes in different field situations. (Modified from Stirling *et al*., 2002.)

Previously uncropped sites	Annual arable crops	Perennial crops and pastures	Vegetables and annual ornamentals	Perennial horticultural crops	Perennial horticultural crops	Turf
To detect nematodes that might affect the crop to be planted.	Preplant sampling to determine whether nematodes may cause problems in the next crop.	Sampling established crops to determine whether nematodes should be controlled.	Preplant sampling to determine whether control measures are needed in the next crop.	Preplant sampling to determine whether nematodes may cause problems after replanting.	Sampling an established crop to determine whether nematodes are causing economic damage and nematicide treatment is warranted.	Sampling to determine whether nematodes are causing economic damage.
Sample intensively and use appropriate extraction procedures as nematode populations are likely to be low and difficult to detect.	Are economically important nematodes likely to be present? The host status of the previous crop is a useful guide.	If there is variability in crop growth, sample poor patches separately from healthy areas.	Consider making observations at the end of the previous crop. For nematodes such as root-knot nematodes, the amount of galling provides an indication of the distribution or density of nematodes and their likely impact on the next crop.	Previous cropping history will provide a good indication of the nematodes likely to be present in a replant situation.	Sample should be collected in the root zone of the crop.	Areas of poor or uneven growth should be sampled separately from healthy areas.
Sample near vegetation rather than in bare soil.	Sample well in advance of planting to allow time for bioassays (e.g. 3–4 months for cereal cyst nematodes).	Collect both root and soil.	Nematode populations decline in fallow soil. Since preplant nematode densities may be low, sampling and extraction procedures must be adequate to detect low numbers of potentially important nematodes.	If the previous crop was trees or vines, collect samples of old roots that may still be harbouring plant-parasitic nematodes.	For some crops roots rather than soil may give a more appropriate sample.	Collect samples from within the root zone (0–15 cm depth).
Ensure that the sample is representative of the plant species present (i.e. sample all weeds, grasses, shrubs and trees).	For sampling root-knot nematodes directly after harvest is an ideal time as densities are highest and thus detection is better.	Observe roots for symptoms of nematode damage.	Collect samples early to allow time for bioassays (e.g. 2 months before planting for root-knot nematodes).	Where plants are to be planted in virgin soil, there may be a greater chance of introducing nematodes on machinery or planting material rather than in the virgin soil.	Limit sampling units to no more than 0.5 ha and ensure samples consist of a minimum of 20 subsamples.	Use a thin corer (less than 10 mm diam.) and collect many small cores rather than a few larger cores (e.g. at least 20 small cores per golf or bowling green).
If trees are present, which might host economically important nematodes, ensure some cores are taken from the root zone.	For potato cyst nematodes, defining sampling time is less essential, as the detection unit (cysts) are less vulnerable to decrease quickly.	For deep rooted crops ensure that some samples are taken at depths where most roots occur.	Limit sampling units to about 0.5 ha and collect no fewer than 20 cores per sample.	There is no point in sampling for nematodes unless such issues are addressed.	For burrowing nematodes on banana, a root disease index may be more useful than a nematode count.	
Collect samples at least 2 months before planting to allow time to bioassay soil for specific nematodes.	Since large areas are involved, cost is an important factor. Consider whether it is possible to sample only areas where nematode problems are most likely.	Collect a composite sample of no fewer than 20 cores.			In some crops, samples should be collected at specific growth stages. For example, on pineapple samples should be collected 12 months after planting and at crop harvest.	

A summary of the aspects to be considered when sampling for plant-parasitic nematodes in various field and crop situations is given in Table 1.1, modified from Stirling *et al.* (2002).

1.3 Sampling in Relation to Phytosanitary Requirements

Countries have often their own phytosanitary regulations resulting in specific requirements for ensuring the pest-free trade products, for example, Canada and USA (for PCN only) (United States Department of Agriculture, 2014) and South Australia (Walker, n.d.).

The requirements relating to nematodes can vary between growing conditions that should be free of plant-parasitic nematode(s) ('nematode free'; soil should be tested or be known to be free of the required species before sowing; or specific nematodes are known to be absent in a country), to yielded products that should be nematode free based on inspection of these products. The latter can be performed by analysis or visual inspection. Host–nematode combinations form a long list and many combinations will only be checked visually. As visual inspection often overlooks nematodes, because of their size and often not causing specific symptoms, prescribed sampling and analysis of products or soil is often a requisite of the trading product.

One of the major quarantine nematodes worldwide is PCN. For seed potatoes many requirements are put in place before the tubers are allowed to go into trade. In the European Union (EU) a specific control directive is active, prescribing sampling of the soil before planting (Anonymous, 2007). EPPO (European and Mediterranean Plant Protection Organization) has protocols describing tests for potatoes before trading is allowed among many other products and the IPPC (International Plant Protection Convention) gives guidelines on sampling of consignments in general (Anonymous, 2008).

Southey (1978) stated that nematodes, especially soil-inhabiting species, are almost impossible to detect by visual inspection procedures. With nematodes, it is rarely feasible to carry out diagnostic work on anything more than the smallest fraction of the whole crop or field. Therefore, negative diagnostic results should be treated with caution and should be viewed as providing a clear indication that the nematode population is below detection level. This is particularly relevant when the same nematode species are prevalent within local production systems. The desired detection limit determines the amount of sampling units to be taken, which means that in extreme situations the whole area, or all of the plants, has to be examined to be sure. In practice 'a rule of thumb' will often be applied and 60 units per ha or consignment will be taken.

1.4 Soil Sampling for Endoparasitic Nematodes

Nematodes that have an endoparasitic stage can be found in the plant/roots at certain times in their development. Most endoparasitic species can be found in the soil and the plant/roots, the numbers can change depending on the developmental stage of both host and nematode. It is therefore important to know when to sample and which matrix to sample for these nematodes, as for instance if sampling of soil takes place when the nematodes are all inside the plants, results might be misinterpreted.

As *Ditylenchus dipsaci* can be found more easily in the product, inspections during the growing season for symptoms on the leaves or sampling the product at maturity are easier than sampling the soil for the presence of nematodes, as these nematodes are highly aggregated in the plant and plant products, whereas in the soil they can be very low in density and therefore difficult to find using soil sampling.

Root-knot nematodes (*Meloidogyne* spp.) spend most of their life cycle inside roots; only the second-stage juveniles and males (when present) can be found in the soil. A detailed description for sampling *Meloidogyne* spp. is provided by Duncan and Phillips (2009). When soil sampling takes place during the growing season, the numbers can be easily underestimated. When soil sampling takes place after harvest, most eggs and juveniles will be associated with root particles present in the soil sample. Underestimation of the density is easily achieved when using the wrong extraction method (den Nijs and van den Berg, 2013) as a number the juveniles are still present in the roots and eggs are lost during the extraction process. Incubation is one way of solving this problem.

Radopholus similis, the so-called burrowing nematode, is an example of an endoparasitic nematode, an important pest to citrus, banana and plantains. It is restricted to tropical regions and causes the toppling disease in banana; it should be sampled by taking roots. For citrus trees the sample should consist of more roots from near the surface (>100 g) than a smaller amount from roots deeper in the soil (Shokoohi and Duncan, 2018); for banana both the finer and heavy roots should be sampled (Coyne *et al.*, 2014).

1.5 Soil Sampling for Ectoparasitic Nematodes

Ectoparasitic nematodes are relatively easy to sample as their life cycle takes place solely in the soil and therefore all stages can be found in the soil. To determine the presence of ectoparasitic nematodes it should suffice to take a soil sample from the area under investigation, taking into account the moisture level of the soil, as this will influence the depth the sample should be taken (see Section 1.2.2). The size of the nematodes dictates the size of the auger to be used (see Section 1.8) and the aim of the sampling dictates the sampling strategy (see Section 1.1).

1.6 Sampling for Entomopathogenic Nematodes from Soil Samples: Isolation and Baiting Techniques

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae are soil-dwelling antagonists of insects. Several species are used commercially in biological control of pest insects in cryptic environments (Grewal *et al.*, 2005). The only free-living stage is the infective dauer juvenile (DJ), which invades insects through natural openings or intersegmental membranes. Once inside the haemolymph. *Steinernema* and *Heterorhabditis* DJ release symbiotic bacteria of the genera *Xenorhabdus* or *Photorhabdus*, respectively, which cause the death of the host within 1–3 days. Approximately 2 weeks after invasion the new-generation DJ exit the cadaver. In the soil environment the cadavers decay rapidly.

Isolation of EPN can have different objectives. Usually, surveys are conducted to find EPN species in a country or certain environments (Stock and Hunt, 2005). Another reason is to understand population dynamics or monitor survival of EPN after application (Susurluk and Ehlers, 2008). To gain a better understanding of EPN populations and their biocontrol potential, a reliable detection method is needed. Several methods for detection are available. A live bait method, the so-called *Galleria mellonella* (Lepidoptera: Pyralidae) baiting method, is most commonly used (Bedding and Akhurst, 1975). The advantage of this method is that it is simple and selective towards EPN and provides information on the general occurrence of a species. It can also be used to estimate the distribution in a field by assessment of positive soil samples within a specific area. *Galleria mellonella* is highly susceptible and most EPN species can be trapped with this lepidopteran insect (Sturhan and Mráček, 2000).

The disadvantage of the baiting method is that information on the population density is not provided. To extract nematodes from soil, the Baermann funnel extraction or centrifugal flotation method are also used (Kaya and Stock, 1997). The quality of data increases with the number of samples per area, which restricts these approaches because of limited available resources or time. As the distribution of EPN populations depends on the distribution of host insects, which is most often highly patchy, the distribution of EPN in a field follows the same pattern. Unless evenly applied by man, nematode populations are highly aggregated (Campbell *et al.*, 1998; Taylor, 1999; Spiridonov *et al.*, 2007), which results in a high uncertainty of population size estimates. Our methodological potential to monitor a population density of EPN is thus very limited. If the significance of EPN in a food web is studied, this limitation is a major drawback. In biological control, the EPN density is certainly also of major interest; however, for the purpose of estimating the biocontrol potential, the percentage of positive soil samples within an area infested with a pest insect population can provide a good indication for the probability of successful control.

1.6.1 Baiting method

If a sampling is conducted to isolate new strains of EPN or survey the occurrence of EPN, then the sampling method does not follow a specific scheme and single samples can be combined. Larger soil samples of approximately 250 g are recommended. If the percentage of positive samples within an area is required, smaller samples are recommended. The following rules should be considered:

- Soil sampling:
  - Taking samples (50–250 g) close to plant roots increases probability for success.
  - Clean instrument between taking each sample.

- Collect soil from at least 10 cm depth and deeper.
- Place each sample in a plastic bag or box.
- Keep at 4–15°C during transportation or storage in the laboratory.
- Do not store longer than 2 days before baiting.
- If necessary, record soil characteristics and vegetation.

- Insect baiting technique:
  - Transfer sample into a hard plastic container.
  - Moisten soil sample if dry to facilitate EPN movement.
  - Add two to five insect larvae, such as *G. mellonella*, *Tenebrio molitor* or other bait insect.
  - Close container and turn upside down.
  - Keep at room temperature. If nematode species active at low temperature are targeted, incubate at 10°C.
  - Remove dead insects after 5 days.
  - Second addition of insects sometimes increases success of isolation.
- Harvest of DJ from cadavers:
  - Put a filter paper into a 5 cm diam. Petri dish.
  - Transfer cadavers onto the filter paper.
  - Put Petri dish into a 9 cm diam. dish.
  - Fill 9 cm dish with tap water just to cover the bottom.
  - Cover the large dish with a lid.
  - After approximately 10 days the DJ emerge from the cadaver and migrate from the filter paper into the tap water in the larger dish (Fig. 1.1).
  - Pour water with DJ into a beaker and fill with fresh tap water.
  - Let nematodes settle to the bottom of the beaker and decant water.
  - Repeat washing steps.
  - Cleaning of DJ suspension can also be done over a 50 µm mesh sieve.
  - Store DJ at 4–15°C (depending on species) in culture bottles with a minimum of water.
  - Use polystyrene tissue culture flasks with canted neck and ventilation cap for storage.
  - Storage in Ringer's solution (7.5 g NaCl, 0.35 g KCl, 0.21 g $CaCl_2 \times 2\ H_2O$ in 1000 ml water) prolongs shelf life.

The baiting method is also described by Orozco *et al.* (2004), including a video with detailed explanations.

The baiting method can also be performed *in situ* in the field to isolate new EPN strains or to monitor persistence of EPN after application. A 10 ml tube (Fig. 1.2) filled with soil and insect larvae is buried 5 cm deep into the soil. A small plant can also be added. The bottom of the tube is closed with a 50 µm plankton

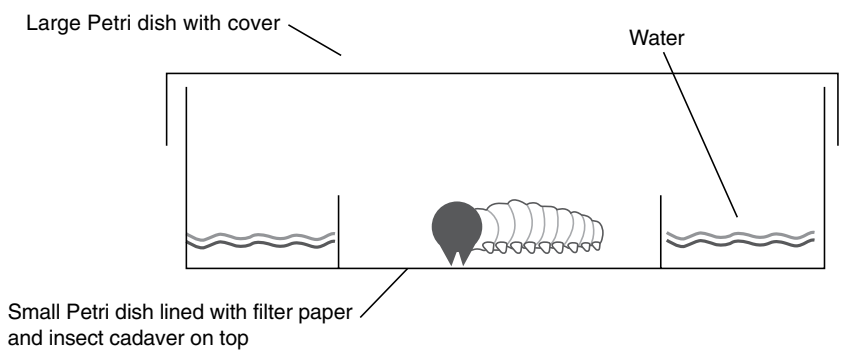

Fig. 1.1. Harvest of dauer juveniles (DJ) of entomopathogenic nematodes from insect cadavers. DJ emerge from the cadaver and migrate from the filter paper into the water in the larger Petri dish.

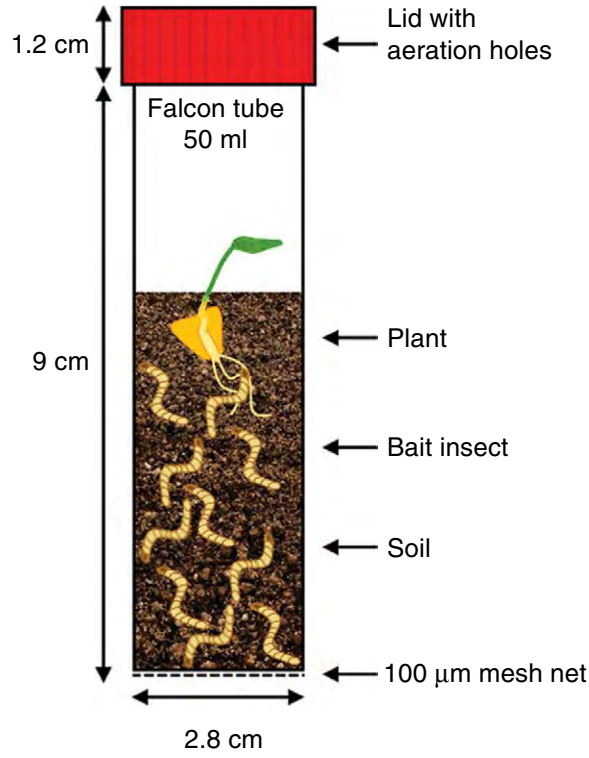

Fig. 1.2. Plastic tube for *in situ* baiting of entomopathogenic nematodes (Bart Vandenbossche, e-nema GmbH, Germany, unpublished).

sieve, so nematodes can easily enter from the bottom, but insects cannot escape. After 7–10 days these tubes are removed and insects can be checked for nematode infestation. Tubes are easily located because of the red lid sticking out 5 cm above the soil surface.

1.7 Examples of Sampling Protocols for Pine Wood Nematodes and Potato Cyst Nematodes

1.7.1 Sampling for *B. xylophilus*

Detection of *B. xylophilus* is difficult in healthy trees. To enhance the chances of finding the nematodes it is recommended to use a risk-based sampling and focus on susceptible trees with a high risk, such as weakened trees caused by forest fire or pathogens other than PWN, as these trees are preferred by the beetle vector, *Monochamus* spp., for oviposition and hence the possibility of nematode transfer. In the warmer climates, searching for trees with symptoms of PWN is possible (EFSA, 2012). Sampling can take place from standing and cut trees, in sawmills and timber yards, or in imported wood, wooden products and solid wood packaging material. Alternatively, the vectors can be monitored by specific insect traps for the presence of the PWN. All actions need different sampling approaches, described in detail by Schröder *et al.* (2009).

If it is suspected that *B. xylophilus* might be present, wood from a standing tree should be collected as follows:

- Before sampling the trunk, the bark must be removed to avoid contamination.
- Use a drilling machine with bits of at least 17 mm (the diameter is not critical but the heat at drilling is and smaller drills may generate more heat).

- Drill slowly to avoid heat development and drill to a depth of up to 4 cm.
- The total amount of wood sampled from the whole tree should be up to at least 60 g, but preferably 100–300 g.
- At each site, sample at least one tree, but preferably five trees; this depends on the number of weakened or dead trees available.
- Avoid cross contamination between samples from different sites by sterilizing the drill/extraction instruments with alcohol and using a mini burner.

1.7.2 Sampling for *G. rostochiensis* and *G. pallida*

In the EU, the detection limit for *G. rostochiensis* and *G. pallida* is set as a 95% chance of finding 1 cyst in a 1500 cm^3 soil sample comprised of 100 cores taken in a rectangular pattern from a 1 ha field containing a hypothetical population of 3.8 million cysts based on the distribution pattern of foci with known length and width gradients (Anonymous, 2007). An extensive analysis on sampling for cyst nematodes can be found in Pickup *et al.* (2018).

1.8 Sampling Tools

Many sampling tools are available such as augers, knives, hand trowels or spades (Fig. 1.3); the purpose of sampling influences the choice of sampling tool (Coyne *et al.*, 2014). For detection and density estimate

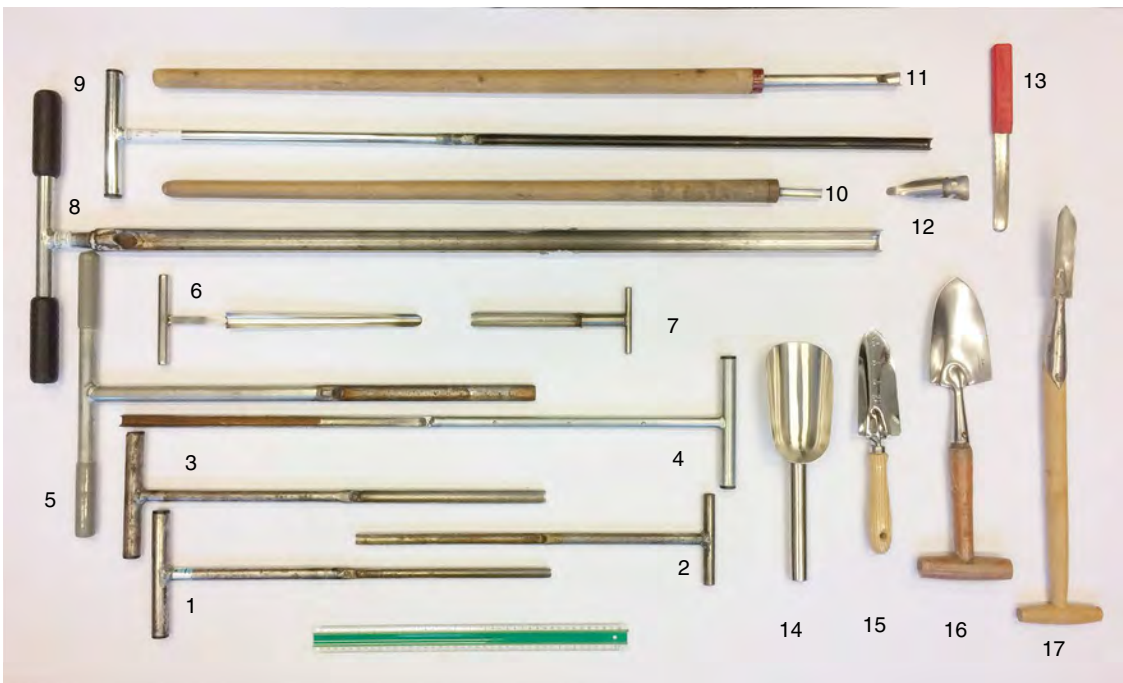

Fig. 1.3. Sampling tools; 1 to 9 are various sizes of augers. 1, 1.0 cm diam., 25 cm length; 2, 1.3 cm diam., 25 cm length; 3, 1.5 cm diam., 25 cm length; 4, 1.2 cm diam., 40 cm length; 5, 2.0 cm diam., 25 cm length; 6, 2.0 cm diam., 25 cm length; 7, 2.0 cm diam., 15 cm length; 8, 3.0 cm diam., 100 cm length; 9, 1.5 cm diam., 60 cm length; 10, 1.0 cm diam., 6 cm length; 11, 2.0 cm diam., 5 cm length. 8 and 9 are special augers for sampling potato cyst nematodes; 12 and 13 are tools for removing soil from augers; and 14 to 17 are small shovels and trowels in various sizes for taking soil and plant samples.

purposes, soil sampling can best be performed using an auger. The size of the nematodes influences the proper choice of the width of the auger: free-living stages of plant-parasitic nematodes can best be sampled using a 15–22 mm diameter auger; when sampling for virus vector nematodes, such as *Longidorus* and *Xiphinema*, an auger with a minimum diameter of 20 mm is best in order to avoid damaging these larger nematodes while sampling. The auger or corer should have a blade length of 20–40 cm. For cyst nematodes some other devices have been developed, such as the 'cheese-sampler' (UK), with a half-cylindrical blade 20–30 cm long and 20–25 mm wide, or the 'Dutch spoon' (automated or by hand), when the depth of the sampling is less important. The depth of the sampling depends on the nematode species and the circumstances, but in general a depth of 20 cm should be enough with a maximum of ploughing depth; when soil cultivation has taken place, cysts can be sampled from up to 5 cm.

1.9 Handling and Storage of Samples

Nematodes are very sensitive due to their small size and absence of skeleton, and when samples are treated incorrectly the nematodes can be damaged or die. It is extremely important that after sampling and before extraction the samples are taken care of properly. If immediate despatch or processing is impossible it is necessary to protect the samples from cold (keep above 4°C) or heat (keep below 27°C), drying out, anaerobic conditions and rough handling as these have a negative effect on the nematodes in the samples (Shurtleff and Averre, 2000). Be aware that nematodes from relatively hot environments can suffer chilling injury (Coyne *et al.*, 2014). All samples sent abroad should be via airmail and include the necessary documentation to clear customs. Transport should preferably take place in an insulated cool box and storage of the samples should be in a refrigerator (4–10°C, depending on the sampling environment) for processing within 2 weeks. For some soil samples, storage time can be extended to 3 to 6 months with only a slight decrease in nematode numbers depending on the species (e.g. numbers of *Meloidogyne hapla* decreased but numbers of *Pratylenchus, Paratylenchus* and *Tylenchorynchus* stayed the same (de Bruin, 1985)).

Samples should always be traceable to the place of sampling, being the field, the lot, the plant/tree. It is of utmost importance that all relevant information is noted and that a label is attached to the sample for recognition. Paper labels should be attached on the outside of the plastic bag, plastic labels could be placed inside the bag. Alternatively, information can be written on the plastic bag. All the necessary information such as sampling date, location and GPS coordinates, crop and cultivar plant species, name of sample taker, name of owner of crop/farmer, a reference number (when more samples are taken on one site), if possible, the previous crop, soil type, treatments and other relevant information should be noted.

1.10 References

Anonymous (2007) Council Directive 2007/33/EC of 11 June 2007 on the control of potato cyst nematodes and repealing Directive 69/465/EEC. *Official Journal of the European Union*, 156/12.

Bedding, R.A. and Akhurst, R.J. (1975) A simple technique for the detection of insect parasitic nematodes in soil. *Nematologica* 21, 109–110. DOI: 10.1163/187529275X00419

Been, T.H. and Schomaker, C.H. (2000) Development and evaluation of sampling methods for fields with infestation foci of potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*). *Phytopathology* 90, 647–656.

Been, T.H. and Schomaker, C.H. (2013) Distribution patterns and sampling. In: Perry, R.N. and Moens, M (eds) *Plant Nematology*, 2nd edn. CAB International, Wallingford, UK, pp. 331–358.

Boag, B. and Neilson, R. (1994) Nematode aggregation and its effect on sampling strategies. *Aspects of Applied Biology* 37, 103–111.

Brown D.J.F. and Boag, B. (1997) Sampling for virus vector nematodes. In: Santos, M.S.N. de A., Abrantes, I.M. de O., Brown, D.J.F. and Lemos, R.M. (eds) *An Introduction to Virus Vector Nematodes and their Associated Viruses*. Instituto Do Ambiente E Vida, Universidade de Coimbra, Portugal, pp. 69–95.

Campbell, J.F., Orza, G., Yoder, F., Lewis, E. and Gaugler, R. (1998) Spatial and temporal distribution of endemic and released entomopathogenic nematode populations in turfgrass. *Entomologia Experimentalis et Applicata* 86, 1–11. DOI: 10.1046/j.1570-7458.1998.00260.x

Coyne, D.L., Nicol, J.M. and Claudius-Cole, B. (2014) *Practical Plant Nematology: A Field and Laboratory Guide,* 2nd edn. SP-IPM Secretariat, International Institute of Tropical Agriculture (IITA), Cotonou, Benin.

de Bruin, N. (1985) Aaltjes in grond tijdens bewaring bij 5° C. In: *Jaarboek 1984 Plantenziektenkundige Dienst, Verslagen en mededelingen nr. 163, 1985*. Plantenziektenkundige Dienst, Ministerie van landbouw en visserij Wageningen, The Netherlands.

den Nijs, L.J.M.F. and van den Berg, W. (2013) The added value of proficiency tests: choosing the proper method for extracting *Meloidogyne* second-stage juveniles from soil. *Nematology* 15, 143–151. DOI: 10.1163/156854112X649954

Duncan, L.W. and Phillips, M.S. (2009) Sampling root-knot nematodes. In: Perry, R.N., Moens, M. and Starr, J.L. (eds) *Root-knot Nematodes*. CAB International, Wallingford, UK, pp. 275–300.

EFSA (2012) Technical assistance on the sampling statistics to be applied pursuant to Commission Implementing Decision 2012/535/EU on emergency measures to prevent the spread of *Bursaphelenchus xylophilus* (the pine wood nematode) within the European Union. Supporting Publications 2012: EN-385. European Food Safety Authority, EU, Brussels, Belgium.

Grewal, P.S., Ehlers, R.-U. and Shapiro-Ilan, D.I. (2005) *Nematodes as Biocontrol Agents*. CAB International, Wallingford, UK.

IPPC (2008) Methodologies for sampling of consignments. Available at: https://www.ippc.int/static/media/files/publication/en/2016/01/ISPM_31_2008_En_2015-12-22_PostCPM10_InkAmReformatted.pdf (accessed 6 August 2018).

Kaya, H.K. and Stock, P. (1997) Techniques in insect nematology. In: Lacey, L.A. (ed.) *Manual of Techniques in Insect Pathology*. Academic Press, San Diego, California, USA, pp. 282–324.

McSorley, R. (1982) Simulated sampling strategies for nematodes distributed according to a negative binomial model. *Journal of Nematology* 14, 517–522.

Orozco, R.A., Lee, M.M. and Stock, S.P. (2014) Soil sampling and isolation of entomopathogenic nematodes (Steinernematidae, Heterorhabditidae). *Journal of Visualized Experiments* 89, 52083. DOI: 10.3791/52083

Pickup, J., Roberts, A.M.I. and den Nijs, L.J.M.F. (2018) Quarantine, distribution patterns and sampling. In: Perry, R.N., Moens, M. and Jones, J.T. (eds) *Cyst Nematodes*. CAB International, Wallingford, UK, pp. 128–153.

Prot, J.C. and Ferris, H. (1992) Sampling approaches for extensive surveys in nematology. *Journal of Nematology* 24(Suppl), 757–764.

Pudasaini, M.P., Schomaker, C.H., Been, T.H. and Moens, M. (2006) The vertical distribution of the plant-parasitic nematode, *Pratylenchus penetrans*, under field crops. *Phytopathology* 96, 226–233. DOI: 10.1094/PHYTO-96-0226

Schröder, T., McNamara, D.G. and Gaar, V. (2009) Guidance on sampling to detect pine wood nematode *Bursaphelenchus xylophilus* in trees, wood and insects. *EPPO Bulletin* 39, 179–188.

Shokoohi, E. and Duncan, L.W. (2018) Nematode parasites of citrus. In: Sikora, R.A., Coyne, D., Hallmann, J. and Timper, P. (eds) *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture*, 3rd edn. CAB International, Wallingford, UK, pp.446–476.

Shurtleff, M.C and Averre III, C.W. (2000) *Diagnosing Plant Diseases Caused by Nematodes*. APS Press, The American Phytopathological Society, St. Paul, Minnesota, USA.

Southey, J.F. (1978) Regulatory controls. In: Southey, J.F. (ed.) *Plant Nematology*, 3rd edn. ADAS, Her Majesty's Stationery Office, London, UK.

Spiridonov, S.E., Moens, M. and Wilson, M.J. (2007) Fine scale spatial distributions of two entomopathogenic nematodes in a grassland soil. *Applied Soil Ecology* 37, 192–201. DOI: 10.1016/j.apsoil.2007.06.009

Stirling, G., Nicol, J. and Reay, F. (2002) *Advisory Service for Nematode Pests – Operational Guidelines*. A Report for the Rural Industries and Development Corporation, Waga, New South Wales, Australia.

Stock, S.P. and Hunt, D.J. (2005) Nematode morphology and systematics. In: Grewal, P.S., Ehlers, R.-U. and Shapiro-Ilan, D.I. (eds) *Nematodes as Biological Control Agents*. CAB International. Wallingford, UK, pp. 3–43.

Sturhan, D. and Mráček, Z. (2000) Comparison of the *Galleria* baiting technique and a direct extraction method for recovering *Steinernema* (Nematoda: Rhabditida) infective-stage juveniles from soil. *Folia Parasitologica* 47, 315–318.

Susurluk, A. and Ehlers, R.-U. (2008) Field persistence of the entomopathogenic nematode *Heterorhabditis bacteriophora* in different crops. *BioControl* 53, 627–641. DOI: 10.1007/s10526-007-9104-2

Taylor, R.A.J. (1999) Sampling entomopathogenic nematodes and measuring their spatial distribution. In: Gwynn, R.L., Smits, P.H., Griffin, C., Ehlers, R.-U., Boemare, N. and Masson, J.-P. (eds). *COST Action 819 Entomopathogenic Nematodes. Application and Persistence of Entomopathogenic Nematodes. Proceedings of a workshop 16–20 May 1995, Todi, Perugia, Italy*. Office for Official Publications of the European Communities, Luxembourg, pp. 43–60.

US Department of Agriculture (n.d.) Guidelines on Surveillance and Phytosanitary Actions for the Potato Cyst Nematodes *Globodera rostochiensis and Globodera pallida*. Available at: https://www.aphis.usda.gov/plant_health/plant_pest_info/nematode/downloads/potato_guidelines.pdf (accessed 27 July 2020).

Walker, G. (n.d.) *A Guide to Soil and Crop Sampling for Nematodes*. Available at: http://www.pir.sa.gov.au/__data/assets/pdf_file/0019/236233/sampling_for_nematodesA.pdf (accessed 27 July 2020).

2 Methods for Nematode Extraction

NICOLE VIAENE,[1,2] JOHANNES HALLMANN[3] AND LEENDERT P.G. MOLENDIJK[4]

[1]*Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Merelbeke, Belgium;* [2]*Ghent University, Ghent, Belgium;* [3]*Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Braunschweig, Germany;* [4]*Wageningen University and Research/Field Crops Lelystad, The Netherlands*

2.1 Introduction

Nematodes can be present in different matrices. Here we describe several methods to extract nematodes from soil and plant parts (Table 2.1). It is crucial that an appropriate method is chosen for the purpose of the research as different types of nematodes, and even different nematode stages, are extracted depending on the method. Factors to consider for choosing the optimal extraction method are the extraction efficiency of the method, the maximum sample size that can be analysed and costs of the extraction equipment. In addition, water consumption, labour and the time needed before nematodes can be examined can be important factors. Results should be evaluated critically in view of the method applied and the circumstances in which the extraction was performed (e.g. equipment, soil type, temperature).

Most methods rely on a combination of three principles inherent to the biology and morphology of nematodes:

- density of nematodes (determines whether they float or sink in water or other liquid);
- size and shape of the nematodes (crucial when sieving); and
- mobility of the nematodes (only certain life stages or nematode types can move out of the matrix).

The most commonly used extraction methods are described in this chapter (Table 2.1), although in practice many variations exist on a same theme. Detailed reviews on extraction techniques for nematodes are available: Oostenbrink (1960), Southey (1986), Seinhorst (1988), van Bezooijen (2006), Ravichandra (2010), Manzanilla-López (2012), Coyne *et al.* (2014) and Hallmann and Subbotin (2018), as well as the European and Mediterranean Plant Protection Organization (EPPO) standard PM 7/119 (1) on nematode extraction (EPPO, 2013). van Bezooijen (2006) and the EPPO standard (EPPO, 2013) also include an indication of the extraction efficiency and costs of each extraction method.

A distinction is made between extraction of cysts from soil and extraction of other nematode forms (vermiform stages and eggs) from soil. This is mainly due to differences in sedimentation rate between cysts and non-cysts, and to the inability of cysts to move. Vermiform nematodes (juveniles or adults) and eggs can occur in soil, but also in plant parts, whilst swollen juvenile stages, typical for some genera, are found only inside roots. Hence, most extraction methods for soil and plant parts overlap once nematodes are set free from plant tissues by cutting or maceration.

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
DOI: 10.1079/9781786391759.0002

Table 2.1. Overview of extraction methods described in this chapter.

Matrix	Type of nematode		
	Cysts	Mobile nematodes	Immobile stages (including eggs)
Soil	Baunacke method Fenwick can Schuiling centrifuge Seinhorst elutriator for cysts Kort's cyst extraction elutriator Wye washer Centrifugal flotation	Baermann method (funnel or dish) Cobb decanting and sieving method Two-flask method Oostenbrink elutriator Seinhorst elutriator Centrifugal flotation	Sieving Centrifugal flotation
Plant		Baermann method Centrifugal flotation	Maceration followed by sieving, or by centrifugal flotation Direct examination Extraction of *Meloidogyne* eggs from roots with egg masses

Methods based on the density of nematodes (also referred to as their specific gravity, i.e. density relative to that of water) rely on the fact that nematodes settle down in a given fluid at a different rate compared to soil and plant particles. This rate of sedimentation depends mostly on their density, but can be influenced by their size and shape (round like cysts or long and vermiform like most free-living stages), their movement (wriggling), as well as by the type of soil in the sample. The time needed for nematodes and soil particles to settle down (sedimentation rate) is associated with the natural gravitational force, but also can depend on forces exerted by an upward current of water or centrifugal forces when using a centrifuge. The average sedimentation rates of some plant-parasitic nematodes were measured by Viglierchio and Schmitt (1983) and ranged between 0.3 cm min^{-1} for *Meloidogyne incognita* second-stage juveniles and 5.2 cm min^{-1} for *Xiphinema index* fourth-stage juveniles and adults. In general, it took longer for small nematodes to settle than for larger nematodes, but sedimentation rates were highly variable, especially for the largest nematodes. The specific gravity of vermiform nematodes is on average 1.08 and varies between 1.04 and 1.09 (Andrássy, 1956). The main exceptions are dried cysts; they have a specific gravity less than 1, hence they float on water. This particular feature is the basis of the extraction methods of dried cysts (see Baunacke, Fenwick and Schuiling methods).

One or more sieves are used at some point in the extraction procedure of most methods. The choice of the size of the sieve, in fact the sieve's pore aperture (expressed in µm or mm), depends on the size (length) of the nematodes one wants to retrieve and the size of the particles to be removed. If the pore aperture is too small, holes can become clogged with soil and plant particles, but rather large holes can result in loss of nematodes. Especially when vermiform nematodes are captured, one has to keep in mind that they can go through the hole 'head first' or 'tail first', as the aperture is larger than a nematode's diameter. A stack of sieves, consisting of three to four sieves with the same aperture placed on top of each other, is often used to reduce this type of loss. Usually sieves with 45 µm apertures are used to obtain most vermiform nematodes (van Bezooijen, 2006), but Byrd *et al*. (1976) and McSorley and Parrado (1981) recommend 38 µm aperture sieves to retain small juveniles of *Rotylenchulus*. Sizes of pore apertures are often expressed in terms of 'mesh', which is related to the number of wires per square inch, but can differ between standard systems applied in different countries (Table 2.2). Note that the smaller the aperture, the higher the mesh of a sieve. Check sieves before use for damage; especially the ones with small apertures should be treated carefully. Before starting, moisten sieves on both sides to avoid clogging. Incline sieves by slightly lifting them and tap on the side of the sieves to help the suspension pass through.

Table 2.2. Conversion tables of sieve sizes, expressed in diameter (µm) of the opening (pore or aperture) and in mesh (related to number of wires per inch) for both American and British standard wire-mesh sieve scales.

Opening size (µm)	US mesh	British mesh
4000	5	4
2800	7	6
2000	10	8
1400	14	12
1000	18	16
850	20	18
840		20
710	25	22
600	30	25
500	35	30
425	40	36
355	45	44
300	50	52
250	60	60
210		72
180	80	85
150	100	100
125	120	120
90	170	170
75	200	200
63	230	240
53	270	300
45	325	350
38	400	400
26	500	

Cysts are the persistent, tanned, survival structures that once were a female but later turned into round or lemon-shaped capsules holding and protecting the eggs of genera such as *Globodera*, *Heterodera*, *Cactodera* and *Punctodera*. For extraction of cysts, several sieve sizes can be used, usually with openings ranging between 250 µm (for *Globodera* spp.) and 100 µm (e.g. for *Heterodera carotae*); use a sieve with apertures that are slightly less than the diameter of the cyst species to be retrieved.

Several methods (e.g. the Baermann method) rely solely, or at some step in their procedure, on the mobility of the nematodes. Mobile stages actively pass through a filter that separates the soil and plant particles from the nematode suspension. Hence, inactive stages, sluggish or dead nematodes are not extracted by these methods. Examples of inactive stages are eggs, swollen nematode stages inside or attached to roots and dauer juveniles. Also, criconematoid nematodes with cuticle appendices like spikes might get stuck in the filter tissue and are also lost by this method. Moving requires energy and time, so the extraction efficiency depends largely on extraction time, but also on temperature and the thickness of the soil or plant layer and the filter that needs to be crossed before the nematodes reach the water (McSorley, 1987).

2.2 Centrifugal Flotation

This method is used to extract mobile as well as immobile nematodes from soil and/or from macerated plant parts (Coolen, 1979). It is also used to clean extracts obtained by sieving or elutriation that still contain soil particles. Light plant particles and other debris that float on water are removed in the first step of the process when centrifuging in water. This step also enables the removal of most of the water from the sample; what

remains are particles heavier than water, including nematodes. Nematode specimens are subsequently separated from the matrix by bringing them in a suspension with a density greater than their own, so they float and denser matrix particles (e.g. soil) sink. Centrifugation is used to speed up the separation of the sinking fraction and floating fraction. The size of the sample that can be processed is limited by the size of the centrifuge tubes.

2.2.1 Materials

- For soil: a subsample obtained after mixing the whole soil sample.
- For plant material: a pair of scissors or knife, blender (e.g. Waring blender, household blender) and a 1200 µm aperture sieve (Coolen and D'Herde, 1972; Coolen 1979; van Bezooijen, 2006).
- Centrifuge and centrifuge tubes with a size ranging between 100 and 1000 ml.
- Extraction fluid: solution with a specific gravity between 1.15 and 1.18, obtained by dissolving $MgSO_4$, sucrose, $ZnSO_4$, colloidal silica (Ludox) or similar in tap water.
- Kaolin: this whte powder is a type of clay that forms a visible layer that separates the solid sediment from the liquid supernatant (water or extraction fluid).
- Stirrer or Vibro mixer.
- 20 µm sieve or smaller.
- Glass beaker.

2.2.2 Procedure

Preparing plant samples:

- Cut the (washed) plant tissues into pieces about 1 cm long. Mix the pieces carefully; if only part of it is used for nematode extraction and take a subsample.
- Macerate the plant tissues, e.g. in a blender at about 12,000 rpm for 30–60 s.
- Pour the resulting suspension through a 1200 µm aperture sieve placed on top of a beaker; collect the water with nematodes.
- Rinse the plant tissues, e.g. on top of the sieve to remove as many nematodes as possible, and collect them in the beaker.

Preparing soil samples:

- In case of clay soil, the sample can be pre-treated by soaking in water to disperse the clay particles (see Southey, 1986).

Centrifuging (Fig. 2.1):

- Quarter fill centrifuge tubes with the soil subsample and/or macerated plant tissues.
- Add water to fill the tubes halfway and add an amount of kaolin (about 1 ml to 100 ml of suspension).
- Stir the suspension thoroughly with a stirrer or Vibro mixer to form a homogenous suspension.
- Centrifuge the tubes for approximately 4 min at 1800 *g*; time and *g* force are not critical, as long as a stable pellet is achieved; time lengths of 2–5 min and *g* forces of 700 to 2900 *g* can be used.
- Gently pour off the supernatant (containing particles floating in water) and discard.
- Re-suspend the pellet in about 400 ml of the extraction fluid, using the Vibro mixer or a stirrer.
- Centrifuge tubes again at 1800 *g* for 4 min.
- Gently pour the supernatant, containing the nematodes, over a 20 µm sieve or smaller.
- Rinse the sieve immediately and abundantly with water to remove the extraction fluid, which has an osmotic effect on the nematodes.
- Transfer the nematodes from the sieve into the glass beaker using water; a clear nematode suspension should be obtained.

Fig. 2.1. **(A)** Schematic workflow of the centrifugal flotation method (after van Bezooijen, 2006). **(B)** Equipment used for centrifugal flotation: 1, centrifuge; 2, balance; 3, centrifuge tubes (1000 ml); 4, container with $MgSO_4$ solution; and 5, Vibro mixer. (Photo: JKI Münster, Germany.)

Advantages: Non-mobile nematodes are extracted; a clean nematode suspension is obtained; nematodes are available for examination within 30 min.
Disadvantages: Some types of extraction fluid can damage nematodes, which hampers recovery and identification; the equipment is expensive.
Remarks: It is recommended that the density of the extraction fluid is checked using a densimeter, as the amount of solute to be dissolved in water can vary with the temperature of the water. The higher the density of the extraction fluid, the more nematodes will be recovered, but the higher the osmotic pressure (depending on the chemical used) the higher the risk of collapsing nematodes (e.g. dorylaimids). Also, when using extraction fluids with high densities more soil particles and debris will be recovered, resulting in dirtier extracts.

Sugar or $MgSO_4$ are cheap but have the highest osmotic pressure, followed by $ZnSO_4$ (but this is acidic and toxic), whilst colloidal silica such as Ludox, Percoll and Ficoll have almost no osmotic effect but are expensive. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) is probably the most commonly used chemical for nematode extraction by centrifugal flotation. Its advantage over sugar is that it is not sticky and can be recycled.

In order to extract the nematodes inside the organic fraction (usually pieces of roots) of a soil sample, the following method is recommended for soil samples (Coolen, 1979; Chen *et al.*, 2000).

- Suspend the soil sample in water and pour through 2 mm and 0.2 mm sieves placed on top of a 1 l beaker, collect all organic material on the 0.2 mm sieve and the large root pieces from the 2 mm sieve, but leave stones on the larger sieve.
- Macerate the organic fraction in a blender with a minimum of water, e.g. at about 20,000 rpm for 1 min (Waring blender, at high speed).

- Pour the resulting suspension through a 840 µm aperture sieve placed on top of the beaker containing the soil, rinse the sieve well but make sure not to exceed 1 l.
- Quarter fill centrifuge tubes with the mineral and macerated organic fraction of the soil sample collected in the 1 l beaker.
- Proceed as described above for centrifugal flotation.

Centrifugal flotation could probably also be used to extract wet cysts; however, this is rarely done, and the method needs to be validated. As dry cysts float on water, they will be discarded with the water after the first cycle of centrifugation using water. They can be collected by pouring this supernatant over a 200 µm sieve. Wet cysts will stay afloat in a separation fluid with specific gravity of 1.28. This higher density is required as the separation fluid can enter cysts that are not completely filled with eggs, hampering their flotation in the extraction fluid with the usual specific gravity of 1.18. The remaining eggs inside the cysts will keep the cysts afloat in the 1.28 fluid. Sometimes cysts are retrieved when centrifuging with a separation fluid of 1.18; these are mainly full cysts.

Centrifugal flotation can also be used to collect nematode eggs from macerated plant tissues containing many eggs (e.g. nematode cultures on carrot discs) (Dunn, 1973; Pudasaini *et al*., 2008). The nematode suspension obtained from the blended tissues, containing eggs and vermiform nematodes, is passed through a 100 µm sieve once, separating eggs from most of the vermiform nematodes. The filtrate is then passed several times through a 50 µm sieve to remove most of the remaining vermiform nematodes, and to obtain mostly eggs in the filtrate. After this series of rinses, the filtrate should be passed through a 20 µm sieve so that the eggs, retained on the top of the 20 µm sieve, can be retrieved.

A variation of centrifugal flotation is the automated zonal centrifugation (Hendrickx, 1995) (Fig. 2.2). Nematodes are extracted from a matrix in one step instead of two. In this process, the sample (soil or mixed plant tissues suspended in water) is gradually added to two layers with different densities (water and separating fluid) inside a centrifuge (15,000–17,000 *g* rpm) consisting of a large bowl. The nematodes are separated from the matrix and move to a layer with a specific gravity between 1.0 (water) and that of the separating fluid, e.g. 1.15–1.20 (e.g. $MgSO_4$). When the spinning slows down, the sediment is sealed off with a layer of kaolin and when it finally stops, the layer with the nematodes, on top of the kaolin, is removed through the central hole in the zonal centrifuge and collected in a beaker. The process can be automated by adding carousels to the

Fig. 2.2. Automated zonal centrifugation. The lower carousel feeds the samples to the centrifuge (1 l beaker) and the upper carousel receives the nematode suspensions (150 ml beaker) after the centrifuging process. The bowl of the zonal centrifuge is kept inside a metal cage for safety. The sample, kaolin (yellow vessel) and $MgSO_4$ (blue container upper right corner) are brought into to the centrifuge bowl through tubes. (Photograph, courtesy ILVO, Merelbeke, Belgium.)

zonal centrifuge for delivery of the beakers with samples and receiving the nematode suspensions. The machine handles samples up to 200 ml of soil, suspended in 1 l water, which results in a 140 ml nematode suspension.

2.3 Extraction of Cysts

There are several methods to extract cysts from soil. Some require dried soil, as they rely on the fact that dry cysts float in water (e.g. Baunacke, Fenwick and Schuiling methods). Other methods do not require the soil to be dry (in fact, the cysts to be dry) (e.g. Seinhorst method, Wye washer, Kort's elutriator), but they can be used for dried soil as well. Most of these methods do not work well for peat soil or other types of soil with a high content of organic matter as the organic particles will float together with the cysts. The best method for such soils is the Schuiling centrifuge (see below and https://www.youtube.com/watch?v=2FJbtrbrd8w). It is also possible to apply a strainer-like plate that pushes large debris down but allows cysts to float through the holes (4 mm diam.) in the plate. Later in the process, the same is repeated with a plate with 1 mm holes. Most cysts will pass through these holes; cyst diameters are less than 1 mm but some cysts of *H. filipjevi*, *H. betae* and *H. avenae* can occasionally be longer than 1 mm. This approach is illustrated for an adjusted and automated Fenwick can used by the Dutch General Inspection Service (NAK) (https://www.youtube.com/watch?v=xhd8GLQzdOA). This equipment is only applicable for dried soil samples, as cysts need to float on water.

When extracting cysts, the aperture size of the sieve(s) used at the end of the process (i.e. to catch the cysts) should be adjusted to the cysts one wants to retrieve. For example, a 250 µm sieve will catch potato cyst nematodes (*Globodera rostochiensis*, *G. pallida*), but a 100 µm sieve should be used for small cysts (e.g. *H. carotae*, *H. urticae*). Cysts (and debris) caught on the collecting sieves are transferred to a filter paper and counted directly using a dissecting microscope, or left to dry before counting on the filter paper or on a counting tray. For some soils, it is possible that lots of debris is extracted together with the cysts. Then it can be useful to clean the cysts from the debris using ethanol or acetone (Seinhorst, 1988).

2.3.1 Baunacke method

This method, developed by Baunacke in 1922, is the easiest to separate cysts from soil. It is also called the 'white bowl method' or 'stirring method'. Buhr (1954) improved the method by using a white paper strip to collect the cysts. Coyne *et al.* (2014) refer to it as the 'sieving method for sedentary cysts'.

2.3.1.1 Materials

- Sample of dried soil.
- Plastic beaker or (white) bowl.
- Stirring rod.
- Paper strip (optional).
- Detergent.
- Pair of forceps, needle or fine painting brush.
- Optionally: sieves with aperture of 3 mm, 1 mm and 250 µm.

*2.3.1.2 Procedure (**Fig. 2.3**)*

- Place the dried soil into the plastic beaker or bowl. If sieves are available, first pass the dried soil with a jet of water through a bank of sieves with 3 mm, 1 mm and 250 µm aperture (or smaller, depending on the cyst of interest) to eliminate as much soil and organic particles as possible; then, transfer the debris remaining on the sieve into the beaker or bowl.
- Stir the suspension thoroughly.
- Let the suspension settle for 30 s to several minutes; depending on the soil type. The water is cleared and the liquid will only contain the floating organic debris and cysts.

- Add a drop of detergent, which causes the cysts to move to the edge of the beaker or bowl.
- Pick the cysts by hand using the forceps, needle or artist's paint brush, under a dissecting microscope.
- A white filter paper strip can be used to make collection of cysts easier. Place the paper strip around the inside of the upper part of the beaker and raise the water level so cysts can adhere to the paper (Buhr, 1954). Carefully remove the strip, now with cysts attached, from the beaker and collect or count the cysts. The strips can also be folded and stored until later for further analysis.

Advantages: Simple, quick and cheap; little water used.
Disadvantages: Sample size is limited to about 100 ml soil; cysts might remain trapped in the soil, so thorough stirring is required; results are strongly dependent on individual operators.

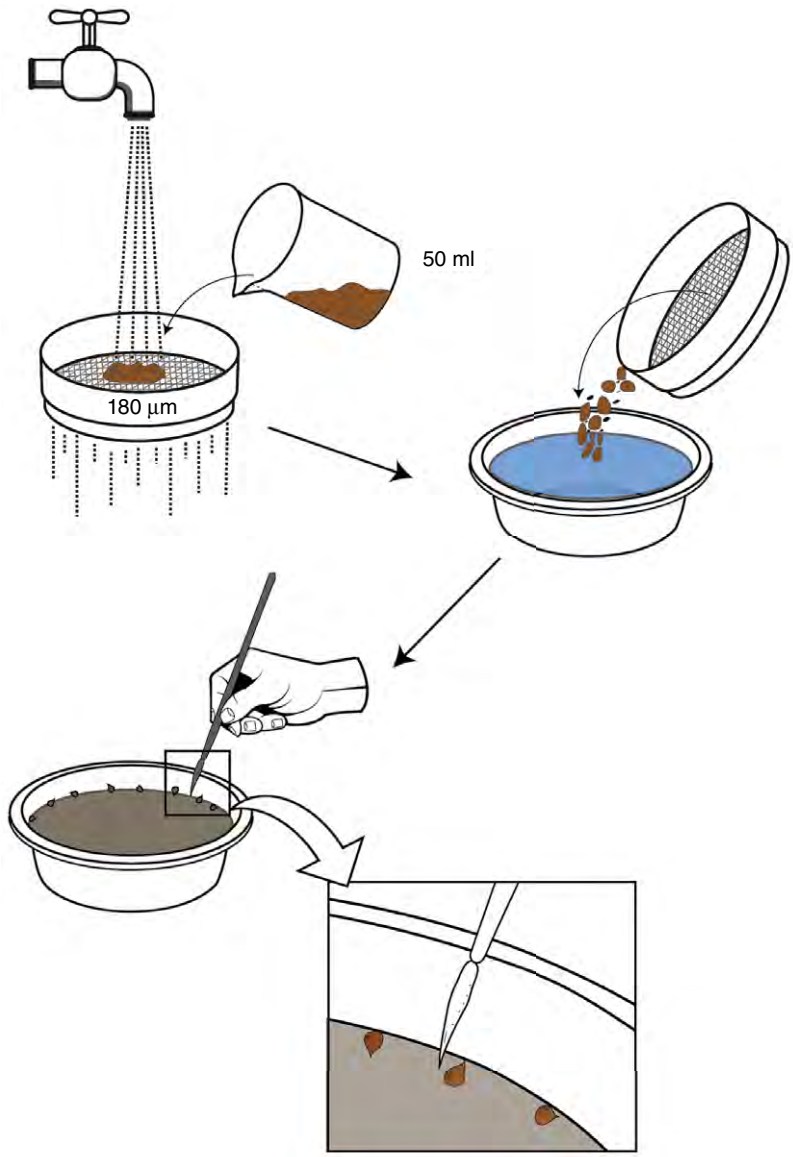

Fig. 2.3. Baunacke method (after van Bezooijen, 2006) using a 180 µm sieve.

2.3.2 Fenwick can

Fenwick (1940) developed a method based on the separation of dry cysts and soil in water. The dry cysts float while the heavier soil particles sink in water. The apparatus uses a water current so that cysts are freed from soil particles and do not have the time to settle. The can is tapered towards the top and has a sloping base, ending in a drain hole that is closed with a plug when the can is in use. Soil is added halfway into the can through a funnel with a long stem (Fig. 2.4). Cysts are separated from soil and move upwards, where they are led, through an overflow collar, to a sieve with small enough aperture to catch them, while the water can pass through.

In an elaborated version, the soil at the base of the Fenwick can is elutriated by water flowing rapidly through a long glass or metal tube inserted deep in the can. This can result in greater cysts recovery as cysts trapped in soil aggregates are freed by the strong water current.

The Fenwick can is used in many laboratories and comes in different sizes, about 30 to 60 cm high. A few places have an automated version comprising several sets of Fenwick cans and sieves, enabling extraction of many soil samples in a short time with a minimum of labour, e.g. the automated carousel of 16 Fenwick can-based extraction units (Fig. 2.5) manufactured by MEKU (www.meku-pollaehne.de) or the extraction unit developed in The Netherlands (see https://www.youtube.com/watch?v=xhd8GLQzdOA&t=22s).

2.3.2.1 Materials

- Fenwick can (Fig. 2.4).
- 1 mm aperture top sieve.
- A sieve with openings of 250 µm, or smaller, to collect the cysts; use a sieve with appropriate aperture to retrieve the type of cysts (see Section 2.3).
- 840 µm sieve (optional).

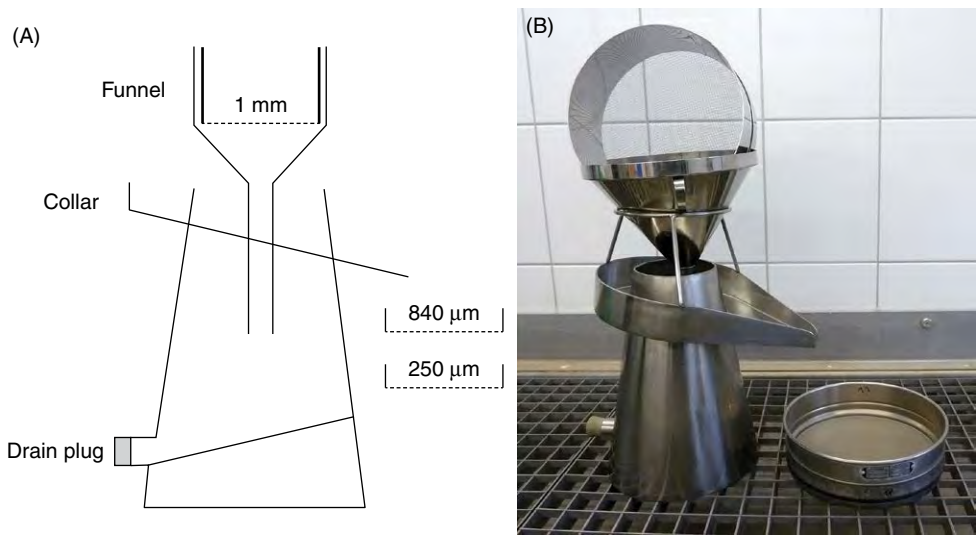

Fig. 2.4. **(A)** Vertical-section diagram of Fenwick can. (Adapted from EPPO Bulletin 39, 354-368: PM 7/40(2), 2009.) **(B)** Fenwick can made of stainless steel with 1 mm top sieve displayed in upright position for better visualization. (Photograph, courtesy JKI Münster, Germany.)

Fig. 2.5. Automated carousel at the Plant Protection Service in Hannover, Germany. (Photograph, courtesy Plant Protection Service, Chamber of Agriculture, Lower Saxony, Germany.)

2.3.2.2 *Procedure*

- Clean the can with water, close the outlet at the bottom and fill the can to the rim with water.
- Place the collecting sieve (250 µm aperture or smaller) under the outlet of the overflow collar.
- Wash the dried soil sample through the top sieve (1 or 2 mm openings) into the can with a strong jet of water; this removes stones and large debris.
- Leave the water running. Heavy soil particles will sink to the bottom of the can, cysts and light root debris will flow out of the can through the collecting collar onto the collecting sieve.
- Let the water flow until the overflowing water is clean, this takes between 1 and 5 min.
- Collect the cysts on the sieve beneath the outlet of the collar.
- An extra sieve with 840 µm openings can be added on top of the collecting sieve to remove large debris that moved with the float; this 840 µm sieve will not retain cysts.
- Rinse the funnel and collar thoroughly to ensure all cysts are gathered on the collecting sieve.
- Remove the stopper at the bottom of the can to remove the remainder of the sample (soil and water); rinse the can as well as the 1 mm top sieve before the next sample is processed.
- Transfer the material collected on the collecting sieve to a filter paper. Rinse the collecting sieve thoroughly before processing the next sample. Alternatively, several collecting sieves can be used and the cysts (and debris) can be left to dry on the sieve before transferring them to a counting dish or for further processing.
- Analyse the cysts and process them for identification using a dissecting microscope.

Advantages: One person can process many samples; easy to construct.
Disadvantages: Soil samples must be dried beforehand; large amounts of water are required.

2.3.3 Schuiling centrifuge

The same principles used for the Fenwick can were applied by Schuiling to develop a semi-automated centrifuge (Hietbrink and Ritter, 1982): dry cysts float while heavier soil particles sink in water.

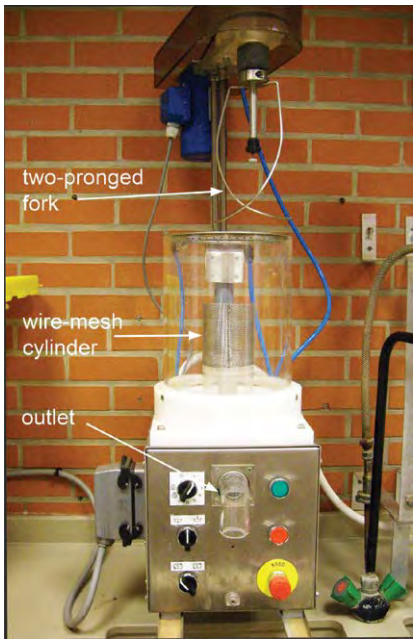

Fig. 2.6. Schuiling centrifuge for the extraction of cysts from dried soil samples. (Photograph, courtesy Centre pour l'agronomie et l'agro – Industrie de la Province de Hainaut, CARAH, Belgium.)

Instead of a water current created by adding water in a long tube inside the can, the water current is created by swirling the mixture of soil and water inside a cylindrical container using a fork (Fig. 2.6). The two-pronged fork is rotated using an electric motor and creates a vortex. The swirling of the mixture forces floating cysts through a mesh in the centre of the container and moves heavy soil particles to the outer parts of the container. The mesh cylinder is fixed above a tube of the same diameter leading to a collecting sieve of 200 µm or 250 µm, or other size depending on the cysts of interest. (Demonstration can be viewed on https://www.youtube.com/watch?v=2FJbtrbrd8w&t=62s.)

2.3.3.1 Materials

- Schuiling centrifuge.
- A collecting sieve, usually a 200 µm or 250 µm aperture sieve.

2.3.3.2 Procedure

- Add up to 500 ml dried soil in the transparent cylindrical container of the apparatus, half filled with water.
- Start the motor, so the content is swirled by the fork, creating a vortex and causing cysts and similar sized floating particles to be forced to the centre and move through a wire-mesh cylinder of 1.5 mm aperture.
- While swirling, more water is added to the inside of the container to wash off any adhering debris or cysts. These cysts can then move to the centre and also pass through the outlet to the collecting sieve.
- The apparatus is automatically cleaned after each sample.

Advantages: Efficacy as high as for the Fenwick can (Bellvert *et al.*, 2008); automated process, so individual failure is low; high throughput of samples; uses less water than for the Fenwick can or Seinhorst elutriator.
Disadvantages: Expensive; samples containing stones and dried clumps of clay may disturb the operation.

2.3.4 Seinhorst elutriator

The Seinhorst elutriator (Seinhorst, 1964) can extract cysts from wet soil as well as dry soil. Similar to the previous methods, this method is based on the difference in density of cysts and soil particles. The cysts are freed from the soil particles by an upward water current created by water entering the base of the can through a perforated tube at a constant rate. The diameters of the tubes and the can and the rate of the water current are such that the sedimentation speed of the cysts is lower than the water current. Soil added to the water at the top of the column falls down in the upward water current whose rate ensures that cysts float and are washed through the overflow, move through a tube and end in a collecting sieve. This sieve usually consists of a bucket with the bottom, and sometimes some side parts, replaced by a mesh of 200 µm or 250 µm aperture, depending on the cysts of interest. Unique to the Seinhorst elutriator is a second collection of cysts that did not reach the collar. These cysts are obtained through a side outlet halfway up the column. This construction results in a minimum efficacy of 98% according to Seinhorst (1964), if operated correctly. An automated version of this technique is available.

2.3.4.1 Materials

- Seinhorst elutriator for cysts (Fig. 2.7).
- 2 mm aperture sieve.
- 200 µm or 250 µm collecting sieve (diam. = 20 cm or bucket with parts replaced by gauze with this aperture).

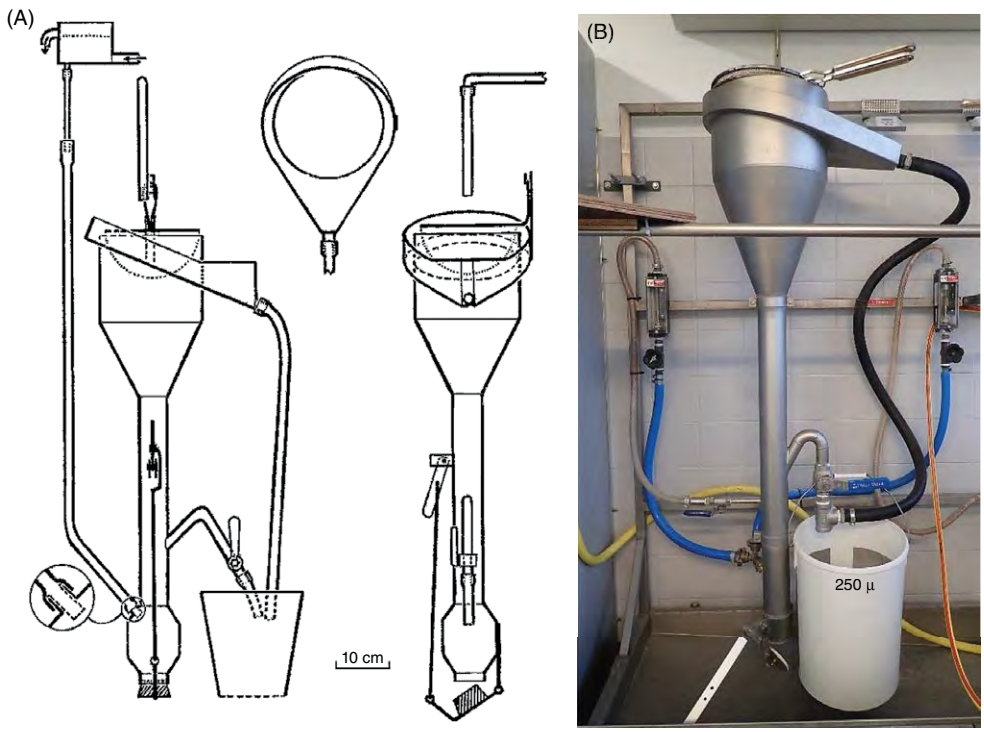

Fig. 2.7. **(A)** Diagram of Seinhorst elutriator for the separation of *Heterodera* and *Globodera* cysts from wet soil. Left: as seen from the side. Centre: as seen from above. Right: as seen from the front. In circle below left: inlet enlarged to show placement of sieve to break the water current. (Adapted from Seinhorst (1964), courtesy *Nematologica*.) **(B)** Scaled-up Seinhorst elutriator for samples up to 2.5 kg. (Photograph, courtesy Wageningen University and Research Centre, Wageningen, The Netherlands.)

2.3.4.2 Procedure

- Place the collecting sieve under both the overflow collar and side outlet.
- Fill the elutriator with water using an undercurrent water stream of 3500 ml min^{-1}.
- When the can is filled halfway (the border between narrow part and funnel-like part), pass the soil sample through a 2 mm sieve into the elutriator.
- Wash the sample into the can while moving the sieve up and down.
- Make sure to add the whole sample before the water starts to overflow.
- Wait for 2–5 min, depending on the soil type, until the water overflow is clean.
- Close the upward water current.
- Rinse the outlet collar with water to ensure all cysts are washed onto the collecting sieve.
- Open the side outlet to add the water in the upper part of the elutriator containing heavier cysts on the 200 µm or 250 µm sieve.
- Collect the cysts from the sieve for immediate investigation or further cleaning.
- Open the outlet at the bottom to release soil and water mixture and clean the elutriator with water.

Advantages: Large samples of up to 1000 ml soil can be handled in a standardized way; no need to dry the soil.
Disadvantages: Expensive equipment; uses a large amount of water; large amounts of debris can accumulate together with the cysts; additional cleaning can be required.
Remarks: A similar elutriator was designed by Kort (1960). Kort's cyst extraction elutriator differs in the shape of the funnel, water flow rate and handling (Fig. 2.8). Been *et al.* (2007) developed a scaled-up version of the Seinhorst elutriator allowing up to 2000 ml (2500 g) of soil to be processed (Fig. 2.7). An automated device is the 'soil sample extractor' made by MEKU (www.meku-pollaehne.de) (Fig. 2.9). The extraction procedure is based on the Seinhorst elutriation principle.

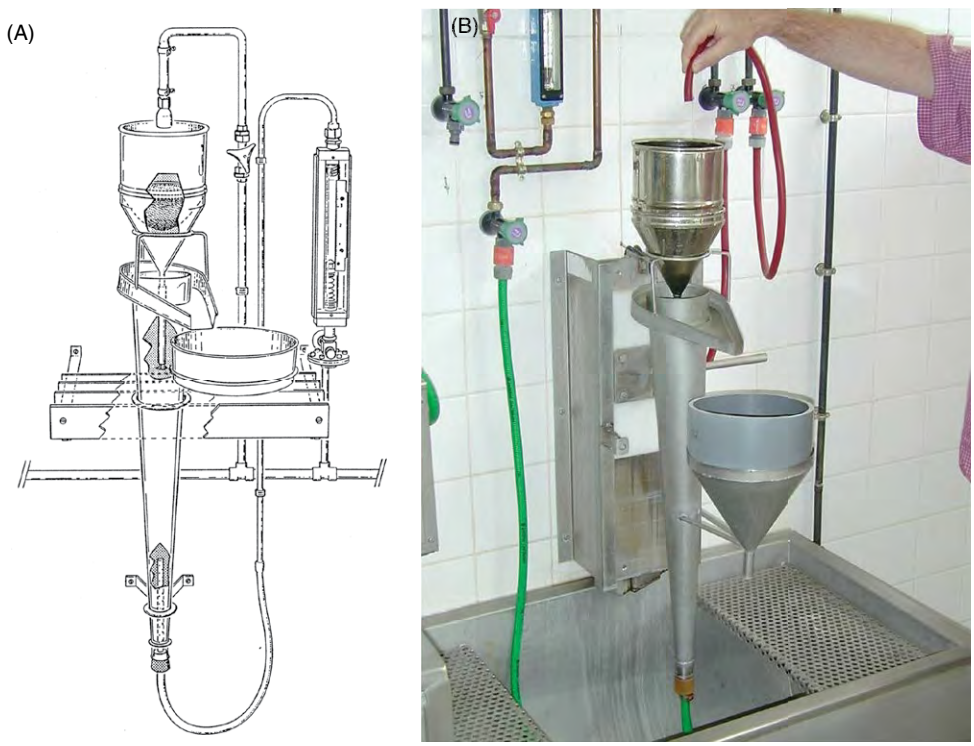

Fig. 2.8. **(A)** Drawing of Kort's cyst extraction elutriator (van Bezooijen, 2006). **(B)** Kort's cyst extraction elutriator in operation. (Photograph, courtesy ANSES-LSV, France.)

Fig. 2.9. Automated soil sample extractor for cysts. (Photo: JKI Münster, Germany.)

2.3.5 Wye washer

The Wye washer (Winfield *et al*., 1987) can be used for the extraction of cysts from wet or dry soil. It is constructed of clear acrylic tube, 50 cm long and 15 cm diam., which is held inside two tight-fitting concentric PVC sleeves at its lower end (Fig. 2.10). Water enters through an inlet pipe on the outer sleeve and is caused to swirl by means of an arrangement of grooves and angled holes on the inner sleeve and the acrylic tube. At the top of the tube is a spout which directs overflow onto sieves that capture the large debris (840 µm aperture) and cysts (100–250 µm sieve), similar to those used with the other extraction devices for cysts.

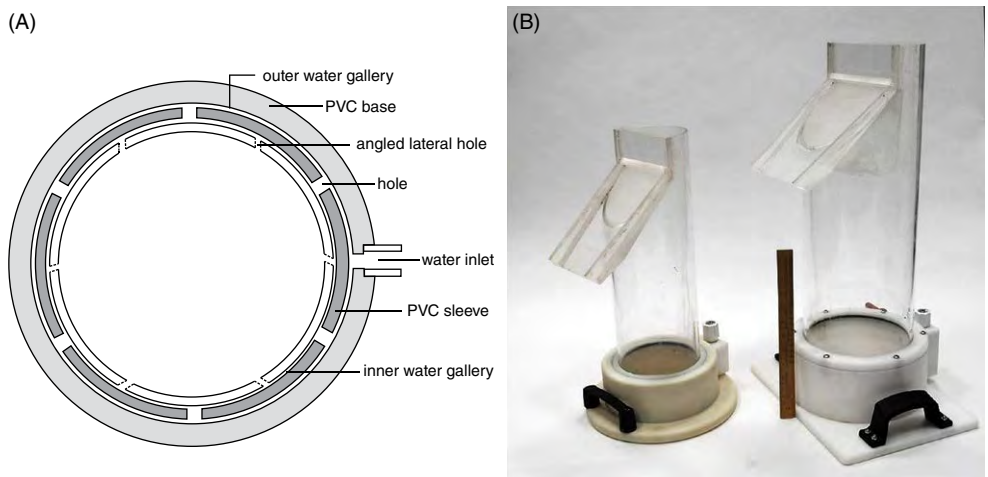

Fig. 2.10. **(A)** Cross section of the base of the Wye washer (after Winfield *et al*. (1986), courtesy *Annals of Applied Biology*). **(B)** Standard Wye washer for samples up to 1000 ml and 'Giant' Wye washer for samples up to 2000 ml. (Photo: FERA, Sand Hutton, UK.)

Fig. 2.11. Wye washer in action (Photo: FERA, Sand Hutton, UK.)

2.3.5.1 Materials

- Wye washer apparatus (Figs 2.10 and 2.11).
- 840 µm sieve.
- 250 µm sieve, or other size, to collect cysts of interest.

2.3.5.2 Procedure

- Add a soil sample (maximum 1 kg for the normal Wye washer, 2 kg for the 'giant' version) to a small quantity of water in the Wye washer.
- Add more water as rapidly as possible, to break up the soil until the rim is reached.
- Briefly stop the flow, then increase water flow gradually to about 10 l min^{-1} for 10 min and let it flow onto the sieves.
- Transfer cysts from the collecting sieve into a glass beaker or other device to process further.

Advantages: Soil samples up to 1 kg or 2 kg can be processed; applicable for wet and dry soil; more consistent recovery of cyst than with the Fenwick can; faster procedure than with the Fenwick can.
Disadvantages: high water use, e.g. 10 min extraction at 10 l min^{-1} = 100 l $sample^{-1}$; high costs as it is a custom-built apparatus.
Remark: The Trudgill tower or Trudgill column elutriator is a similar device that is also used to extract cysts from soil, and in particular cysts still attached to roots. A description of the Trudgill tower can be found in Manzanilla-López (2012) and Trudgill *et al.* (1973).

2.4 Extraction of Vermiform Nematodes from Soil

The following methods rely on the mobility of vermiform nematodes. The only method to extract non-mobile nematode stages from soil is the centrifugal flotation method (see Section 2.2).

2.4.1 Baermann method

This widely used method, fit for soil as well as plant samples, is fairly simple and can be used in any laboratory as it requires no sophisticated material. The principle of this extraction method, described by Baermann (1917), has been applied to several designs, generally making use of a filter (cloth, paper tissue, cotton-wool) to separate mobile nematodes from the matrix (soil and/or plant parts), which is held on top of a supporting basket or coarse sieve. The matrix is just barely immersed in water, so nematodes can move from soil to water, where they sink to the bottom of the recipient holding the water. The whole set-up is put in a dish (also called tray) or a funnel with a closed stem. The funnel allows the collection of all nematodes in a small volume of water as they sink into the stem; hence only a few ml need to be removed from the stem. With a dish, all the water in the dish needs to be transferred to a beaker for further examination.

Baermann (1917) introduced the method using a funnel; Oostenbrink (1954) modified it and called it the 'Oostenbrink dish' or 'cotton-wool filter method'. It is also known as the Whitehead and Hemming tray (Whitehead and Hemming, 1965), or simply the Whitehead tray method or extraction tray method (Coyne *et al*., 2014). Using a pie pan, with sloping sides and flat bottom, facilitates pouring out the water with nematodes and resulted in the name 'pie pan method'. Several modifications exist and most laboratories have their own design of the Baermann method, differing in type and size of the recipient, the support system and the filter (Southey, 1986; Ryss, 2017). When the supporting sieve is rather small, e.g. a 9 cm Petri dish or even a sieve fitting into a microtube (Eppendorf type), it has been referred to as the 'micro-Baermann method'.

2.4.1.1 Materials

- Baermann funnel: Glass funnel (preferably not plastic as nematodes stick to this surface), ideally with a steep slope (approx. 30°). A piece of soft polyethylene tube should be attached to the stem and closed with a clip (spring or screw type), or clothes peg (cloth pin) if nothing else is available. A stand to hold the funnel is also needed (Fig. 2.12), but if a mistifier is used for extraction of nematodes from plant parts (see Section 2.5.3), this is part of the equipment (Figs 2.12 and 2.13).
- Baermann dish: plastic or stainless-steel dish (pie pan); size can vary (Fig. 2.13).
- Support, such as plastic sieve or wire basket with large enough aperture to allow passage of nematodes (i.e. 250 µm). This can also be made of a short piece plastic cylinder with a nylon gauze glued to one end (Fig. 2.13B).

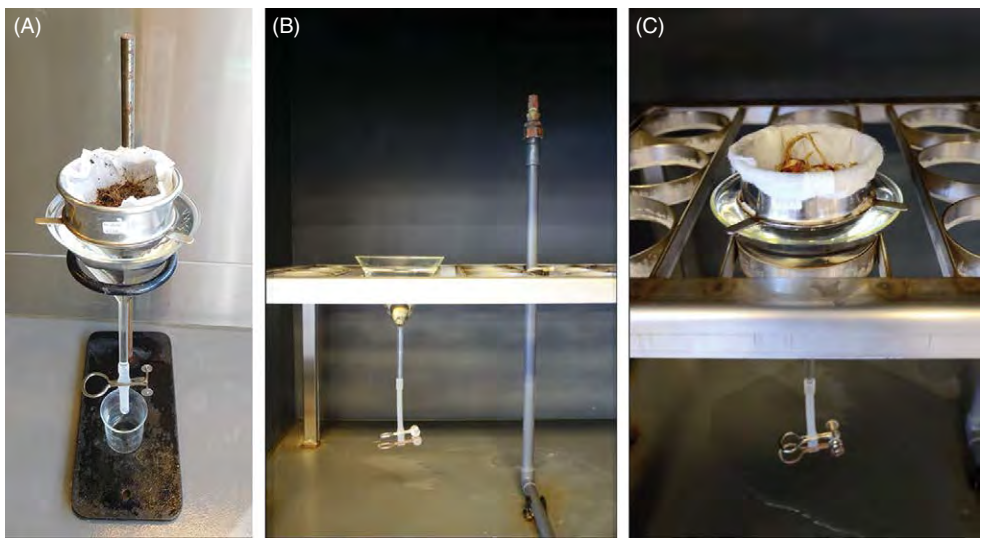

Fig. 2.12. Baermann funnel for extracting nematodes from plant material or soil, here depicted with plant parts. **(A)** Held in a stand. **(B)** Placed in a mistifier, next to the mist sprayer. **(C)** Upper view in the mistifier. (Photo: ILVO, Merelbeke, Belgium.)

- Filter strong enough to hold the matrix, permitting passage of nematodes but not of soil or plant particles: cotton-wool filter (as used to filter milk), fleece, paper filter, one or two layers of paper towel. Nematode passage can vary greatly depending on filter material and thickness of filter. The filter goes on top of the support.
- If plant tissues are used (see also Section 2.5.3): a knife, pair of scissors or blender will be needed to cut the tissues.
- 20 µm sieve.
- 100 ml glass beaker.

2.4.1.2 Procedure

- If working with plant material, cut the plant tissues in ±1 cm pieces, this improves release of nematodes inside the tissues.
- Place soil or plant material on the filter placed within the support (sieve). One can omit the filter for plant materials that are clean and do not have small particles that can enter the nematode suspension.
- Add water to the funnel or dish.
- Submerge the support gently in the water of the funnel/dish. The matrix should also be in contact with the water, at least always touching the water surface, but never totally submerged in water. When using a tray, the support holding the matrix should be raised just slightly (a few mm) above the bottom of the tray to allow nematodes to move from the matrix to the water; it should never touch the bottom. When not working in a mistifier apparatus, make sure there is always contact with water: if water evaporates fast, cover with a lid or add water when necessary; do not let the matrix dry out.
- Nematodes leave the soil or plant tissues, pass through the filter and sink to the bottom of the funnel stem or dish.

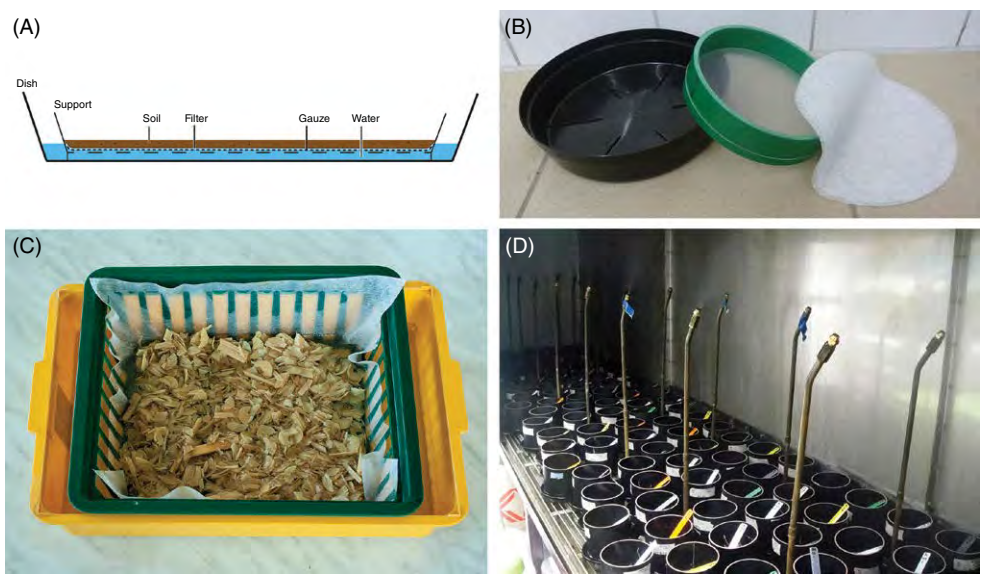

Fig. 2.13. Baermann dish. **(A)** Schematic drawing of a Baermann dish (adapted from Southey, 1986). The dish can have any size. The support does not rest on the bottom of the dish, but hangs or stands on legs. It often contains a wire gauze with coarse openings that allow smooth passage of nematodes. Support and gauze are usually attached to each other, making up one piece. Water added to the dish should barely touch the thin layer of soil or plant parts, not flood it. **(B)** Set-up showing plastic dish, supporting sieve made of polyamide gauze and cotton-wool milk filter. (Photo: JKI Münster, Germany.) **(C)** Set-up consisting of plastic dish, plastic basket and cotton-wool milk filter for extracting *Bursaphelenchus xylophilus* from wood chips. (Photo: Vladimir Gaar, Diag. Lab. Prague, Czech Republic.) **(D)** Set-up in a mist-chamber. (Photo: Wageningen University and Research Centre, Wageningen, The Netherlands.)

- Collect nematodes in a glass beaker, usually after 24 h, by opening the spring or screw clip on the funnel stem or by collecting the nematodes of the dish. The time varies with the aim of the extraction, type of material and nematodes of interest.
- Let the nematodes settle in the glass beaker and remove the supernatant, or pass the suspension over a sieve small enough to retain the nematodes on the sieve, to reduce the volume of water.
- Ideally extraction should be at about 22°C.

Advantages: Simple and inexpensive; uses small amount of water; final suspension is clean; good recovery of mobile nematodes from small samples; when viable eggs are present in the sample (e.g. *Meloidogyne*), nematodes can be recovered for several days to weeks.
Disadvantages: Poor recovery of relatively non-mobile nematodes (e.g. *Xiphinema*, *Hemicycliophora*, *Criconemoides*); poor recovery from thick layers of soil samples (the layer of soil should be 2–3 mm; when extracting from plant materials in a mistifier, the layer of plant tissue can be 1–2 cm); without a mistifier, there can be lack of aeration in the water, which reduces nematode movement and, hence, recovery (adding fresh water every 1–2 days can help improving nematode recovery); some plant material (e.g. bulbs, potato peels) provoke fast bacterial growth, especially after maceration, resulting in turbid suspensions, bad odours and dead nematodes; time needed to obtain nematodes is long (at least 24 h).

2.4.2 Cobb decanting and sieving method and the Flegg-modified Cobb method

The flotation and sieving technique is a simple, yet adequate, method to obtain nematodes from a soil sample. It was developed by Nathan Cobb, the pioneer of nematology in the USA (Cobb, 1918). A set of good-quality sieves with different apertures is the only special equipment required. Soil is repeatedly washed in water, decanted and nematodes are collected on sieves, of which the size varies during the process and can differ according to the goal of the extraction. Using sieves of different apertures allows the collection of nematodes belonging to different types (sizes) and removes much of the soil particles during the process. However, the decanting and sieving can be followed by cleaning the suspension with the Baermann technique, or similar, based on nematode mobility. Thus, the method makes use of differences in size, shape and sedimentation rate between nematodes and soil particles, and of nematode mobility.

2.4.2.1 Materials

- Beaker of about 2 l.
- Stirring rod.
- Set of sieves with decreasing apertures, e.g. six sieves with openings of 1 mm, 710 µm, 250 µm, 150 µm, 90 µm, 63 µm).
- Two large glass beakers or bowls (4 l), with diameter larger than that of the sieves.
- Watch glass (6 cm diam.).
- Baermann funnel/Oostenbrink dish (see Section 2.4.1).
- 100 ml glass beaker(s).

2.4.2.2 Procedure

- Put 200 ml of soil in a 2 l beaker and add about 1 l of water.
- Stir the soil suspension vigorously for 10 s to suspend all particles and free the nematodes.
- Allow the soil to settle for 15 s.
- Pour the supernatant through the 1 mm diam. sieve into a large beaker or bowl, leaving behind the heavy material in the first beaker; discard this settled material.
- Rinse the material on top of the sieve carefully over the large beaker; nematodes going through the sieve end up in the beaker. The nematodes on top of the sieve are collected in a 100 ml beaker.

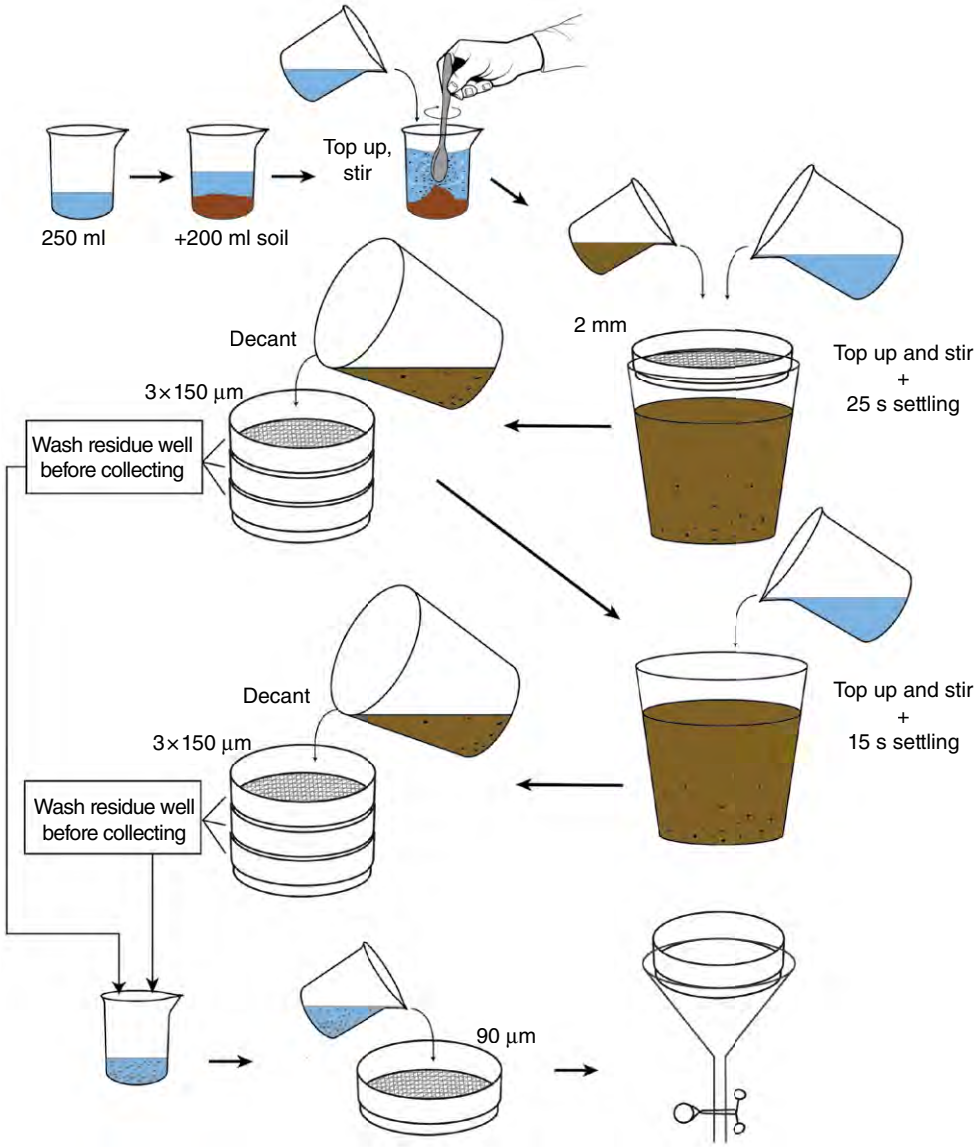

Fig. 2.14. Scheme of the modified Cobb's washing, decanting and sieving technique. (After Flegg, 1967.)

- Repeat the procedure with sieves of decreasing apertures, rinse what goes through the sieves into a large beaker (or bowl) and what stays on top of the sieve into a 100 ml beaker. All nematodes can be added to one 100 ml beaker; if nematodes of different sizes are to be kept apart, then separate beakers can be used.
- Carefully pour the suspension from the 100 ml beaker, with the help of a watch glass or spoon to spread the suspension, onto the filter in the Baermann funnel/Oostenbrink dish.
- Add water until the bottom of the filter is just covered.
- After 24 h, collect the nematodes from the funnel or dish in a glass beaker.

Advantages: No elaborate apparatus needed; high extraction efficiency; little use of water; mobile stages of all nematode genera can be recovered; rapid and simple.
Disadvantages: Maximum of 200 ml soil; not suitable for clay soil because light colloidal particles remain in suspension; due to the final cleaning step with the Baermann technique, only mobile stages of nematodes are recovered; labour intensive; operator experience required to carry out the method in a reliable way.
Remarks: The Flegg-modified Cobb technique (Flegg, 1967) is a variation on the Cobb method of washing, decanting and sieving for extraction of mobile nematodes from soil, and is especially recommended for the extraction of large dorylaimid nematodes (e.g. *Xiphinema*, *Longidorus*). This method makes use of three nested sieves with 150 µm apertures, instead of consecutively using a set of sieves with decreasing aperture size (Fig. 2.14).

2.4.3 Two-flask method

The two-flask method, also referred to as the Erlenmeyer method or milk bottle method (Fig. 2.15), is the predecessor of the Oostenbrink and Seinhorst elutriators but is easier to construct. With this method, active nematodes are extracted from soil based on differences in sedimentation rates of nematodes and soil particles, as well as on nematode mobility to clean the obtained nematode suspension (Seinhorst 1955, 1962, 1988). A bottle with a relatively wide opening is filled with a soil suspension and turned upside down on top of another similar bottle, which is completely filled with water. As soil particles from the upper bottle sink faster than nematodes into the lower bottle, and water from the lower bottle moves up into the upper one, soil and nematodes are separated. Also, the upward water stream replacing heavy soil particles prevents sinking of nematodes and other light particles.

2.4.3.1 Materials

- Three bottles with a wide opening, e.g. milk bottles (1 l, maximum sample size 100 ml) or Erlenmeyer flasks (2 l, maximum sample size 500 ml) (note: fragile!), labelled A, B and C.
- Stand with ring and clip.
- Domestic sieve (2 mm aperture).
- A large funnel, with a plug, to add the soil into the first bottle.

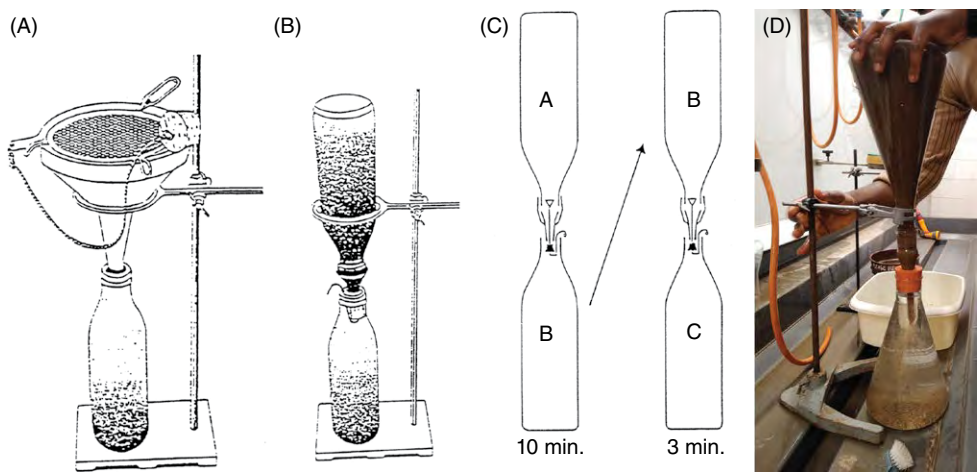

Fig. 2.15. Two-flask method (drawings from J. van Bezooijen). **(A)** Step 1: sieving soil into water of bottle A. **(B)** Step 2: sedimentation where bottle A is placed on top of bottle B for 10 min. **(C)** Step 3: bottle B turned and placed on top of bottle C (3-min sedimentation). **(D)** The two-flask method just after the upper flask was placed upside down, showing the heavier soil particles falling into the lower bottle.

- A home-made 'junction' that can be placed between two bottles: a type of funnel with a cork stopper (Fig. 2.16).
- 45 µm sieves.
- Plastic bowl (4 l).
- Baermann dish or funnel (see Section 2.4.1).
- 100 ml beaker.

2.4.3.2 Procedure

- Close the funnel with the cork stopper, hang it in the ring on the stand and fill it with water. Place bottle A below the funnel. Put the domestic sieve in the closed funnel and add the soil sample through the sieve into the water (Fig. 2.15; step 1). Unplug the funnel, releasing the soil suspension into bottle A. Rinse the funnel and the sieve with water until bottle A is completely filled. Attach a closed junction to bottle A.
- Fill bottle B with water and place it in a stand. Thoroughly shake bottle A and place it upside down in the ring on the stand, so that the closed end of the junction is hanging in bottle B. Quickly open the junction and leave the set-up for 10 min. Heavy soil particles and some of the nematodes sink from bottle A into bottle B. (Fig. 2.15; step 2). Close bottle A and keep it to one side.
- Repeat the procedure with bottle B, placing it on a water-filled bottle C but for only 3 min this time. After this, nematodes will still be afloat in bottle B but bottle C will contain mostly soil particles (Fig. 2.15; step 3). The content of C can be discarded.
- Empty bottles A and B on the set of 45 µm sieves, wash the debris on top of the sieves into the 4 l bowl, rinse both sides of the sieves and let this the suspension settle for at least 5 min.
- Carefully transfer the whole nematode suspension from the bowl, via a watch glass, on the filter of a Baermann dish or funnel and incubate for 16–48 h.
- Bring the clean nematode suspension from the dish (using a water bottle with clean water) or from the funnel into a 100 ml beaker for further analysis.

Advantages: Easy to construct; a high extraction efficiency can be obtained; only small amounts of water are needed and no running water is required.
Disadvantages: Labour intensive; time consuming.

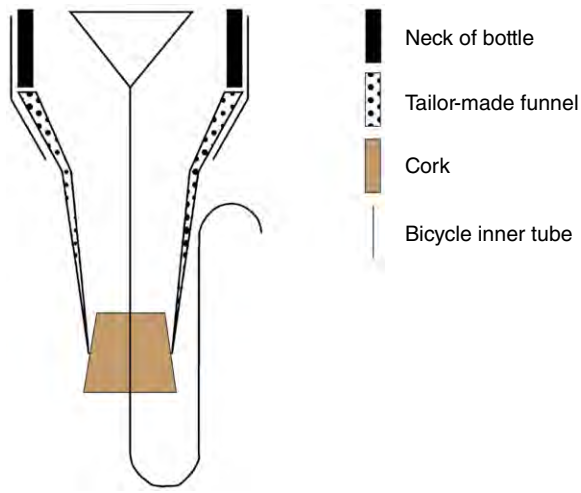

Fig. 2.16. Junction (funnel part) used for the two-bottle method. (After van Bezooijen, 2006.)

2.4.4 Oostenbrink elutriator

The Oostenbrink elutriator (Oostenbrink, 1960) is used in many laboratories to extract mobile nematodes from soil samples. It is based on the principle of flotation using an upward current of water (elutriation). The current is such that nematodes cannot sink, but soil particles do. Other elutriaton methods have been described by Seinhorst for vermiform and cyst nematodes (see Section 2.3.4). Following the elutriation, the nematode suspension is put on a Baermann funnel or Oostenbrink dish to obtain a cleaner nematode suspension as nematodes move through the filter and soil particles remain. This method allows the extraction of soil samples between 100 and 500 ml, depending on the soil type.

2.4.4.1 Materials

- Oostenbrink elutriator (made of stainless steel or acrylic glass) (Figs 2.17 and 2.18).
- Two water supplies: a nozzle to add water at the top of the elutriator, and a water inlet through the bottom via a flowmeter.
- Flowmeter.

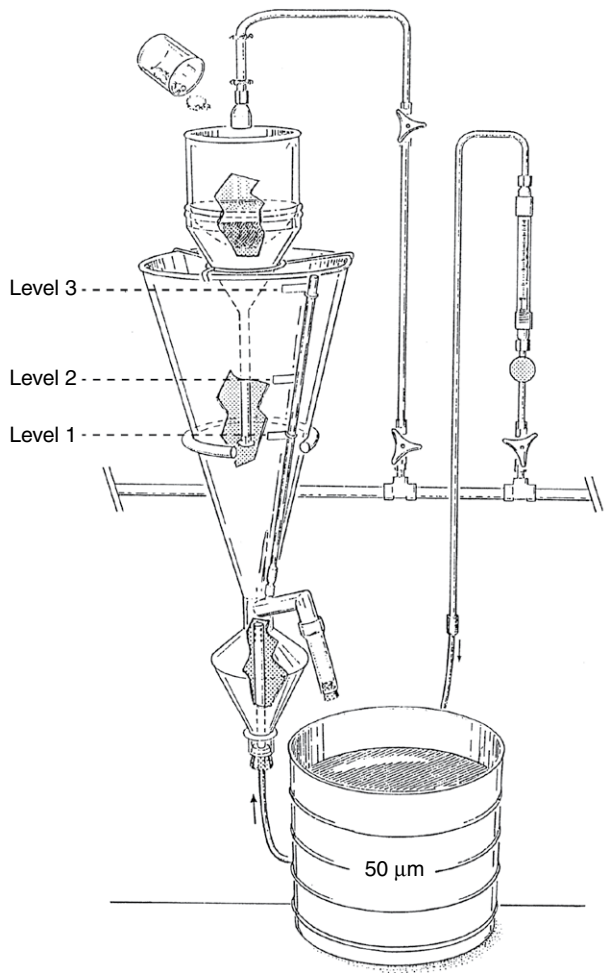

Fig. 2.17. Schematic overview of the Oostenbrink elutriator. (Courtesy van Bezooijen, 2006.)

Fig. 2.18. Oostenbrink elutriator made of stainless steel with stack of four sieves. (Photo: ILVO, Merelbeke, Belgium.)

- 1 mm aperture sieve (or 2–4 mm aperture for large nematodes).
- Set of 4 × 45 µm or 4 × 50 µm aperture sieves (can be adapted according to the size of the nematodes of interest).
- Plastic bowl.
- Baermann funnel/Oostenbrink dish and watch glass (6 cm diam.).

2.4.4.2 Procedure

(See: https://www.youtube.com/watch?v=t9WUkkfoEvs.)

- Close the outlet on the side and the one at the bottom of the elutriator.
- Add water in the funnel, using the inlet (bottom) and the nozzle (top), up to level 1 (water just touches the stem of the funnel).
- Set the undercurrent water stream at 1000 ml min^{-1}.
- Wash the sample through the top sieve (1 mm) into the funnel using the nozzle.
- Continue until the water level reaches level 2, i.e. when the funnel is about two thirds full.
- Close the nozzle, so no more water is added from above.
- After a few seconds, reduce the undercurrent to 600 ml min^{-1}; let the undercurrent fill the funnel.
- Place the set of three or four 45 or 50 µm aperture sieves under the side outlet.
- When the water reaches level 3 (almost at the rim of the elutriator), open the side outlet and let the suspension run on the four nested sieves.
- Immediately wash debris off all sieves into a plastic bowl.
- Transfer the content (slightly dirty nematode suspension) to the Baermann funnel or Oostenbrink dish to remove dirt from the nematode suspension or use centrifugation.

Advantages: Efficient; easy to standardize.
Disadvantages: Expensive equipment; labour cost when not automated; high water consumption.
Remarks: The maximum sample size is determined by the fact that the soil must be washed into the funnel in the time span when water rises from level 1 to level 2. This depends largely on the soil type. In the original

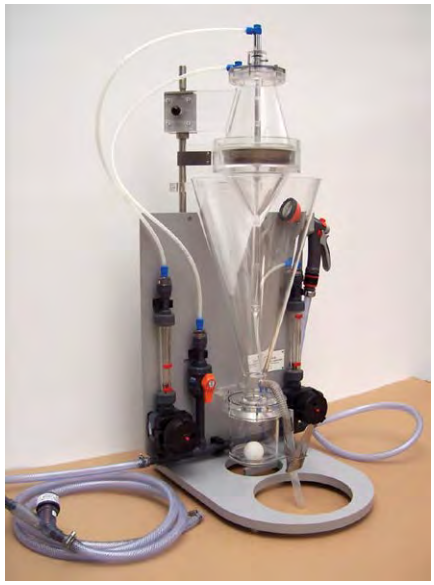

Fig. 2.19. Modified Oostenbrink elutriator made of acrylic glass. (Photo: MEKU GmbH, Germany.)

description (Oostenbrink, 1960; Seinhorst, 1988), level 1 is lower than the tip of the stem of the funnel (as shown in Fig. 2.17); the water current is set to 1000 ml min^{-1} and the soil sample is added as soon as the water reaches the small pipe in the middle of the apparatus (where the side outlet is connected). This gives more time to add the soil sample, but the recovery of nematodes is reduced as many nematodes are immediately brought into the lower part of the elutriator (below the side outlet) and turbulence there is not sufficient to separate them from the heavier soil particles. An extraction takes about 10–15 min.

The water flow can be reduced below 600 ml min^{-1} if the extraction is aimed at small nematodes (e.g. *Paratylenchus*); for larger nematodes (e.g. *Xiphinema*, *Longidorus*) it can be increased to 1500–2000 ml min^{-1}. Also, for larger nematodes three nested sieves of 160–200 µm are recommended.

Nematodes from the organic fraction of the soil (mainly roots) are not extracted. They can be recovered by incubating the organic fraction on Baermann dishes in a mistifier for up to 4 weeks. This organic fraction is retrieved by washing the soil sample over a 180 µm sieve, for example. The organic debris can be extracted separately or combined with the mineral fraction that was recovered after elutriation. Nematodes extracted after 72 h are considered to be from the mineral fraction, while nematodes retrieved after that are considered to originate from the organic fraction.

The Oostenbrink elutriator can be purchased from https://www.mirma.nl/projecten/nematode-apparatuur or http://www.meku-pollaehne.de/Nematologie/Oostenbrink-Elutriator/oostenbrink-elutriator.html (Fig. 2.19).

2.4.5 Seinhorst elutriator

The Seinhorst elutriator (Seinhorst, 1988) is used to extract all stages of mobile nematodes from the mineral fraction of soil. This does not include cysts, nematodes residing inside the organic fraction of the soil (e.g. root pieces), eggs or dead nematodes. Nematodes are separated from the soil particles using an upward water current (elutriation) and subsequently caught in separate containers through different outlets. Each outlet corresponds with a certain range of nematode sizes and soil particles. The collected nematode suspensions are subsequently cleaned by passing them several times through a bank of 50 µm sieves (Seinhorst, 1988). Alternatively, a combination of sieving and the Baermann method can be applied to clean the suspension. The result is a very clear nematode suspension that is fit for further analysis.

2.4.5.1 Materials

- 2 l flask with mouth piece and stopper (Fig. 2.20).
- A 1 mm sieve (or household sieve).
- Seinhorst elutriator (Figs 2.21 and 2.22).
- Bank of sieves (50 µm aperture, 25 cm diam.).
- Extraction dish with filters (see Baermann method, Section 2.4.1).

2.4.5.2 Procedure

- Wash the soil sample (up to 1000 ml) through a 1 mm sieve (to remove stones and root pieces), hung on top of a funnel placed above a 2 l flask.
- Collect the suspension into a 2 l flask (A) and close the flask with a small funnel-shaped mouthpiece (turned with steel upwards) that fits perfectly on the opening of the flask (Fig. 2.20). Close the end of the steel of this small funnel with a stopper so that flask and fitting funnel are leak proof. The mouthpiece is constructed in such a way that the stopper can be removed easily, opening flask A once it is installed in the elutriator (see below).
- Shake the flask gently while holding the stopper firmly and turning the flask upside down until all the contents are mixed.
- Fill the Seinhorst elutriator with water using a constant water supply of 80 ml min^{-1} via tube K (Fig. 2.21).

Fig. 2.20. **(A)** A set of six flasks with soil samples ready to be added on top of five improved Seinhorst elutriators. (see Figs 2.21–2.22). (Photo: Wageningen University and Research Centre, Wageningen, The Netherlands.) **(B)** The funnel-shaped mouthpiece with a stopper to close a flask before it is put upside down on the elutriator, where, once installed, it can be opened easily to release its content. (Photo: Wageningen University and Research Centre, Wageningen, The Netherlands.)

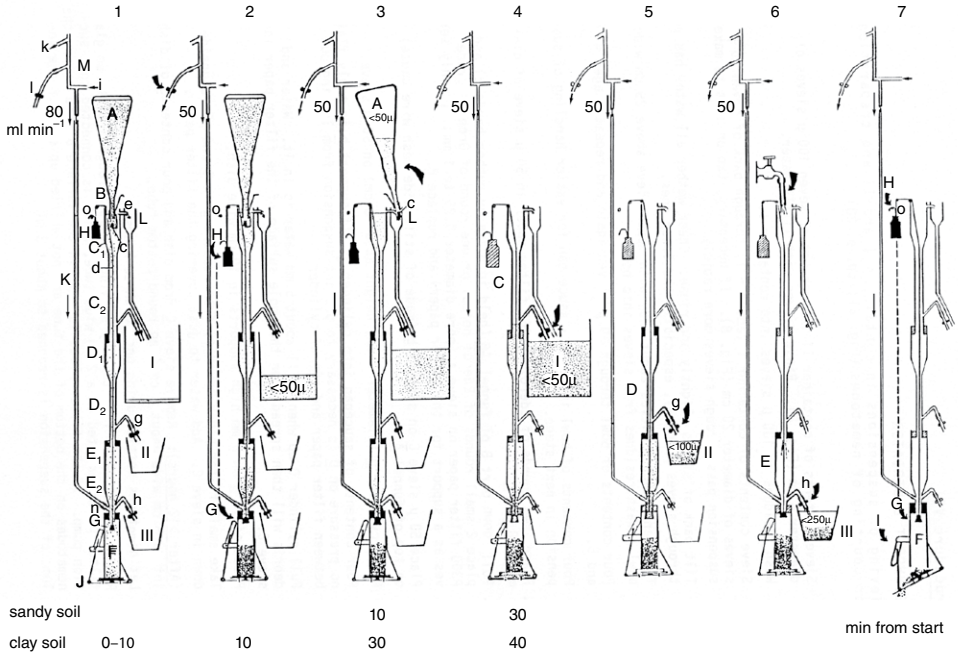

Fig. 2.21. A scheme of the steps involved in the functioning of the Seinhorst elutriator (1988). (Courtesy of Seinhorst, 1988.)

Fig. 2.22. A series of six identical improved Seinhorst elutriators, each with three buckets (I, II, III, top to bottom) and flasks installed on top. (Photo: Wageningen University and Research Centre, Wageningen, The Netherlands.)

- When the water reaches the top of section C1 of the system (Fig. 2.21) and starts flowing into bucket I, place flask A, with thoroughly mixed contents, upside down on top of funnel B of the elutriator (Fig. 2.21, step 1).
- Open flask A by detaching the stopper c (Fig. 2.21, step 1). At this stage, soil particles can be seen moving steadily downwards.
- This is the start of the elutriation process. As the 80 ml min^{-1} flow rate is greater than the sedimentation rate of the larger particles in the suspension, no particle > 50 µm is accumulated in bucket I (Fig. 2.21) (i.e. larger soil particles and nematodes sink). Hence, bucket I contains very small soil particles (diameter < 50 µm) and small, light nematodes (< 1.3 mm or slightly longer, but very thin ones).
- After 10 min, the flow rate is reduced to 50 ml min^{-1} (Fig. 2.21, step 2). This allows particles > 50 µm diameter to pass through C2, of which the larger ones (between 100 and 250 µm), sink further through D to E.
- After 20 min from the start, empty the contents of flask (A), now holding only particles < 50 µm, into bucket I (Fig. 2.21, step 3).
- The contents of the elutriation tube (C in Fig. 2.21) is allowed to flow to bucket (I) for the next 10 min, while the flow rate is kept at 50 ml min^{-1}.
- After 30 min of elutriation, empty the contents of tube C into bucket I by opening the corresponding siphon (f) (Fig. 2.21, step 4).
- Close the siphon (f), refill the tube (up to C2) and rinse twice, to clean the apparatus and the contents of the tube C. Empty these two rinses in bucket I.
- Buckets II and III are filled in a similar way by opening siphons g and h, respectively. This empties tube D, containing soil particles < 100 µm and nematodes of 1–2 mm, and tube E, with the heavier and larger nematodes, e.g. *Xiphinema*, *Trichodorus* and particles with diam. 100–250 µm (Fig. 2.21, steps 5 and 6).
- Finally, after closing the siphons (f, g, and h), open the receptacle (F) (Fig. 2.21, step 7) to remove the sand sedimented during the elutriation process and drain the whole elutriator.
- The nematode suspensions obtained in the separate buckets contain nematodes of different sizes, as well as small soil particles. The volume of the nematode suspensions collected in the buckets is then reduced by passing through a bank of sieves (50 µm, 100 µm and 250 µm for buckets I, II and III, respectively), and collecting the nematodes on top of the sieves.
- Transferring these nematodes to a filter on a Baermann dish or funnel will further clean up the nematode suspensions.

Advantages: Clean suspensions; possibility to split nematode suspension in separate parts based on nematode size.
Disadvantages: Labour intensive; time required for one sample is 40–50 min depending on the soil type (however, one person can operate six elutriators at a time); high water use; high initial costs as the Seinhorst elutriator is a custom-built apparatus.
Remarks: The Seinhorst elutriator was adapted at Wageningen University and Research Centre, Wageningen, The Netherlands, to capture small sized nematodes in a more efficient way: > 90% of *Meloidogyne* juveniles and > 80% of *Pratylenchus penetrans* are retrieved, using a bank of seven 50 µm sieves. A detailed description of the method can be obtained from Teklu *et al.* (2018).

2.5 Extraction of Nematodes from Plant Parts

2.5.1 Direct observation

The simplest way to extract nematodes from plant tissues is by direct observation with a dissecting microscope teasing apart the plant tissues in a small amount of water in a Petri dish. This way, the presence of females or egg masses, or other stages of certain genera of plant-parasitic nematodes (e.g. seed gall nematodes, *Anguina* spp.) can be observed and nematode specimens can be removed for further examination with a compound microscope if needed.

2.5.2 Incubation

Another simple way to extract mobile nematodes is to incubate pieces of chopped plant tissues (roots, wood pieces, bulbs, seeds) in a plastic bag or closed glass jar or flask with some water at the bottom. Adding 1–3% H_2O_2 for oxygenation can increase the efficacy of extraction (Tarjan, 1967). It is best to shake the container occasionally to prevent nematodes from re-entering the plant tissues. After 24 h, migratory stages of endoparasitic nematodes can be found in the water. Immersing the plant pieces in water and shaking the container continuously, e.g. on a shaking platform, is also possible. The water in the container is collected after a minimum of 24–48 h and can be inspected immediately. If this suspension is too voluminous or contains too much dirt, it can be reduced in volume and cleaned using centrifugal flotation or a Baermann funnel.

2.5.3 Baermann method and mistifier

When extracting nematodes from plant parts (roots, bulbs, tuber peels, stems, seeds, leaves, bark, wood), the Baermann devices (funnel, dish; see Section 2.4.1) are often placed in a mistifier. A mistifier is a confined chamber where small droplets of water are released into the air creating a humid environment for the plant tissues, providing a water film in which nematodes can move after leaving the plant tissue. Spraying a fine mist of water on top of the plant materials increases movement of nematodes out of the tissues as a constant water film is provided (and there is no risk of plant parts drying out). The overflowing water in the funnel or dish washes away toxic products of certain types of plant tissues, and the spray supplies oxygen to the nematodes. Care should be taken that the support, holding the plant tissues, is not immersed too deep in the dish or the funnel; it might even be kept a few millimetres above the water surface as nematodes are washed with the drops into the water. If placed too deep, the dish or funnel will be filled with water (provided by the mistifier), and nematodes will not fall to the bottom, but be rinsed over the edge.

The water temperature of the mist or spray should be around 20°C to allow optimum mobility of all nematode specimens. The fine spray is best provided intermittently (e.g. 5 min spraying every 30 min), to save on water. Hollow cone or solid cone nozzles are generally used, releasing water at 4–6 l h^{-1} under a pressure of about 2.8 kg cm^{-2}. In case of fog spray, nozzles are best placed at the sides of the chamber, not above the funnels, spraying into the interior of the apparatus. A fog spray equipment runs constantly. When left for too long (> 4 weeks), bacterial cultures can flourish. This can result in unclear nematode suspensions and even can clog the tubing and filter. Complete refreshment of the water in the funnel or tray every 3–4 days improves extraction efficacy.

2.5.4 Maceration

Maceration, either mechanically using a blender or chemically by enzymatic digestion (Araya and Caswell-Chen, 1993; Viaene *et al*., 2007), will release more nematodes than simply chopping the plant tissues. Macerated plant tissues can be further processed using the Baermann technique or centrifugal flotation to obtain clean nematode suspensions.

2.5.5 Eggs

Separate eggs of *Meloidogyne* can be extracted from roots containing egg masses by shaking the roots (with egg masses) vigorously for 4 min in a sodium hypochlorite (NaOCl) solution (0.53–1.05%) (Hussey and Barker, 1973), followed by extensive rinsing, over a stack of sieves to remove the chlorine. Retrieve the eggs on a 20 µm sieve. If the egg suspension is still dirty due to small soil particles, it can be cleaned by centrifugal flotation. Separated eggs obtained this way facilitate counting nematode numbers in reproduction assays. The eggs can still be used as an inoculum, provided the chlorine was washed away quickly and completely after shaking the roots in the bleach solution.

2.6 Acknowledgement

The authors thank Dieter Slos for his help in improving the drawings of several of the extraction techniques in this chapter.

2.7 References

Andrássy, I. (1956) Die Rauminhalts- und Gewichtsbestimmung der Fadenwürmer (Nematoden). *Acta Zoologica, Budapest* 2, 1–15.

Araya, M. and Caswell-Chen, E.P. (1993) Enzymatic digestion of roots for the recovery of root-knot nematode developmental stages. *Journal of Nematology* 25, 590–595.

Baermann, G. (1917) Eine einfache Methode zur Auffindung von Anklyostomum (Nematoden) Larven in Erdproben. *Geneeskundig Tijdschrift voor Nederlandsch-Indië Batavia* 57, 131–137.

Been, T., van Beers, T., van Bekkum, P. and Beniers, A. (2007) A scaled-up Seinhorst elutriator for extraction of cyst nematodes from soil. *Nematology* 9, 431-435. DOI: 10.1163/156854107781351990

Bellvert, J., Crombie, K. and Horgan, F.G. (2008) Comparative efficiency of the Fenwick can and Schuiling centrifuge in extracting nematode cysts from different soil types. *Journal of Nematology* 40, 30–34.

Buhr, H. (1954) Untersuchungen über den Kartoffelnematoden - I. Die „Papierstreifen-Methode", ein vereinfachtes Verfahren zur Untersuchung von Bodenproben auf ihren Besatz mit Nematodenzysten. *Nachrichtenblatt für den Deutschen Pflanzenschutzdienst* 8, 45–48.

Byrd Jr, D.W., Barker, K.R., Ferris, H., Nusbaum, C.J., Griffin, W.E., Small, R.H. and Stone, C.A. (1976) Two semi-automatic elutriators for extracting nematodes and certain fungi from soil. *Journal of Nematology* 8, 206–212.

Chen, S., Hendrickx, G. and Moens, M. (2000) The importance of organic matter when assaying *Meloidogyne chitwoodi* soil populations. *Russian Journal of Nematology* 8, 147–152.

Cobb, N.A. (1918) *Estimating the Nema Populations of Soil*. USDA Technical Circular 1, Washington D.C., USA.

Coolen, W.A. (1979) Methods for the extraction of *Meloidogyne* spp. and other nematodes from roots and soil. In: Lamberti, F. and Taylor, C.E. (eds) *Root-knot Nematodes (*Meloidogyne *species) Systematics, Biology and Control*. Academic Press, London, UK, pp. 317–329.

Coolen, W.A. and D'Herde, C.J. (1972) *A Method for the Quantitative Extraction of Nematodes from Plant Tissue*. State Agriculture Research Centre, Ghent, Belgium.

Coyne, D. L. Nicol, J. M. and Claudius-Cole, B. (2014). *Practical Plant Nematology: A Field and Laboratory Guide*, 2nd edn. IITA, Cotonou, Benin.

Dunn, R. (1973) Extraction of eggs of *Pratylenchus penetrans* from alfalfa callus and relationship between age of culture and yield of eggs. *Journal of Nematology* 5, 73–74.

EPPO (2013) EPPO standard PM 7/119 (1) on nematode extraction, 2013. *EPPO Bulletin* 43, 471–495. DOI: 10.1111/epp.12077

Fenwick, D.W. (1940) Methods for the recovery and counting of cysts of *Heterodera schachtii* from soil. *Journal of Helminthology* 18, 155–172. DOI: 10.1017/S0022149X00031485

Flegg, J.J.M. (1967) Extraction of *Xiphinema* and *Longidorus* species from soil by a modification of Cobb's decanting and sieving technique. *Annals of Applied Biology* 60, 429–437. DOI: 10.1111/j.1744-7348.1967.tb04497.x

Hallmann, J. and Subbotin, S.A. (2018) Methods for extraction, processing and detection of plant and soil nematodes. In: Sikora, R.A., Coyne, D., Hallmann, J. and Timper, P. (eds) *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture*, 3rd edn. CAB International, Wallingford, UK, pp. 87–119.

Hendrickx, G. (1995) An automatic apparatus for extracting free-living nematodes stages from soil. *Nematologica* 41, 308. (Abstr.)

Hietbrink, H. and Ritter, C.E. (1982) Separating cysts from dried soil samples by a new centrifugation and a flotation method. In: *Proceedings of XVIth International Symposium, European Society of Nematologists*, 30 August – 3 September, St Andrews, UK, pp. 28–29.

Hussey, R.S. and Barker, K.R. (1973) A comparison of methods of collecting inocula, including a new technique. *Plant Disease Reporter* 57, 1025–1028.

Kort, J. (1960) *A Technique for the Extraction of* Heterodera *Cysts from Wet Soil and for the Estimation of their Egg and Larval Content*. Verslagen en Mededelingen van de Plantenziektenkundige Dienst Wageningen No 233, Wageningen, The Netherlands.

Manzanilla-López, R.H. (2012) Methodology and symptomatology. In: Manzanilla-López, R.H. and Marbán-Mendoza, N. (eds) *Practical Plant Nematology*. Biblioteca Básica de Agricultura, Colegio de Postgraduados and Mundi-Prensa, Guadalajara, Jalisco, Mexico, pp. 89–129.

McSorley, R. (1987) Extraction of nematodes and sampling methods. In: Brown, R.H. and Kerry, B.R. (eds) *Principles and Practice of Nematode Control in Crops*, Academic Press, Sydney, Australia, pp. 13–41.
McSorley, R. and Parrado, J.L. (1981) Effect of sieve size on nematode extraction efficiency. *Nematropica* 11, 165–174.
Oostenbrink, M. (1954) Een doelmatige methode voor het toetsen van aaltjesbestrijdingsmiddelen in grond met *Hoplolaimus uniformis* als proefdier. *Mededelingen Landbouwhogeschool Gent, Belgium* 19, 377–408.
Oostenbrink, M. (1960) Estimating nematode populations by some selected methods. In: Sasser, J.N. and Jenkins, W.R. (eds) *Nematology: Fundamentals and Recent Advances with Emphasis on Plant Parasitic and Soil Forms*. The University of North Carolina Press, Chapel Hill, North Carolina, USA, pp. 85–102.
Pudasaini, M.P., Viaene, N. and Moens, M. (2008). Hatching of the root-lesion nematode, *Pratylenchus penetrans*, under the influence of temperature and host. *Nematology* 10, 47–54. DOI: 10.1163/156854108783360078
Ravichandra, N.G. (2010) *Methods and Techniques in Plant Nematology*. PHI Learning Private Limited, New Delhi, India.
Ryss, A.Y. (2017). The simplest 'field' methods for extraction of nematodes from plants, wood, insects and soil, with additional description how to keep extracted nematodes alive for a long time. *Parazitologiia* 51, 57–67.
Seinhorst, J.W. (1955) Een eenvoudige methode voor het afscheiden van aaltjes uit grond. *Tijdschrift over Plantenziekten* 61, 188–190. DOI: 10.1007/BF02651811
Seinhorst, J.W. (1962) Extraction methods for nematodes inhabiting soil. In: Murphy, P.W. (ed.) *Progress in Soil Zoology*. Butterworths, London, UK, pp. 243–256.
Seinhorst, J.W. (1964) Methods for the extraction of *Heterodera* cysts from not previously dried soil samples. *Nematologica* 10, 87–94. DOI: 10.1163/187529264X00664
Seinhorst, J.W. (1988) *The Estimation of Densities of Nematode Populations in Soil and Plants*. Växtskyddsrapporter No 51, Uppsala, Sweden.
Southey, J.F. (1986) *Laboratory Methods for Work with Plant and Soil Nematodes*, 6th edn. Her Majesty's Stationery Office, London, UK.
Tarjan, A.C. (1967) Influence of temperature and hydrogen peroxide on the extraction of burrowing nematodes from citrus roots. *Plant Disease Reporter* 51, 1024–1028.
Teklu, M.G., Beniers, J.E., Been, T.H., Schomaker, C.H. and Molendijk, L.P.G. (2018) *Methods for the Estimation of Partial Resistance in the Glasshouse of Potato Cultivars/Genotypes against Potato Cyst Nematodes and Root-knot Nematodes*. Technical Report, Wageningen University and Research, Wageningen, The Netherlands. DOI: 10.13140/RG.2.2.30798.56648
Trudgill, D.L, Evans, K. and Faulkner, G. (1973) A fluidizing column for extracting nematodes from soil. *Nematologica* 18 (1972), 469–475. DOI: 10.1163/187529272X00070
van Bezooijen, J. (2006) *Methods and Techniques for Nematology*. Available at: https://www.wur.nl/upload_mm/f/9/3/10aac0cb-8289-400a-a6e5-c4687461d138_MethodsandTechniquesforNematology.pdf (accessed 21 July 2020).
Viaene, N., Mahieu, T. and de la Peña, E. (2007) Distribution of *Meloidogyne chitwoodi* in potato tubers and comparison of extraction methods. *Nematology* 9, 143–150. DOI: 10.1163/156854107779969637
Viglierchio, D.R. and Schmitt, R.V. (1983) On the methodology of nematode extraction from field samples: Baermann funnel modifications. *Journal of Nematology* 15, 438–444.
Winfield, A.L., Enfield, M.A. and Foremann, J.H. (1987) A column elutriator for extracting cyst nematodes and other small invertebrates from soil samples. *Annals of Applied Biology* 111, 223–231. DOI: 10.1111/j.1744-7348.1987.tb01449.x
Whitehead, A.G. and Hemming, J.R. (1965) A comparison of some quantitative methods of extracting small vermiform nematodes from soil. *Annals of Applied Biology* 55, 25–38. DOI: 10.1111/j.1744-7348.1965.tb07864.x

3 Estimating Numbers

JOHANNES HALLMANN[1], MATTHIAS DAUB[2] AND WIM WESEMAEL[3,4]

[1]*Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Braunschweig, Germany;* [2]*Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Elsdorf, Germany;* [3]*Flanders Institute for Agriculture, Fisheries and Food (ILVO), Merelbeke, Belgium;* [4]*Ghent University, Ghent, Belgium*

3.1 Introduction

Estimating nematode numbers is required to quantify the presence of certain nematode taxa in plant tissue, field soil or any other substrates. A wide range of applications exist, such as for the evaluation of plant–nematode interactions (root penetration, invasion biology, induction of feeding sites), characterizing nematode populations or host plant reactions (virulence types, resistance screening), nematode management (threshold densities, multiplication rates, suppressiveness), biodiversity studies (environmental indicators, key species) and behavioural studies (movement, attraction, survival). However, enumeration is not always required. For example, in international trade the detection of a single quarantine species is sufficient to take measures and reject the associated consignment.

In most cases, nematode numbers are estimated in liquids that result from the extraction procedures described by Viaene *et al*. (Chapter 2, this volume). The cleanliness of the liquid varies depending on the tissue that was extracted (e.g. plant tissue, field soil, organic substrates) and sometimes additional purification steps are required. There are different enumeration techniques for different nematode stages, such as for eggs, vermiform stages, swollen females, cysts or galls. Depending on the purpose, estimation can relate to the total population or only a fraction of it, such as the viable or non-viable part of a population. The latter is especially crucial in case of quarantine issues, where only viable specimens are relevant.

Estimation of sedentary nematodes (e.g. *Meloidogyne*, *Globodera*, *Heterodera*, *Punctodera*) usually requires an additional step. Besides extracting the cysts or galls/egg masses from the soil or plant, they need to be further processed to release the eggs and juveniles, which then will be counted.

Counting is generally done using optical or light microscopes. There are different microscope types available for different tasks. The most common one is the compound microscope consisting of an objective lens above the nematodes (up to 100×) and eyepiece lenses (10×). Although this allows a maximum magnification up to 1000×, it is not suitable for counting nematodes in suspensions. The height of the nematode suspension requires shorter and thus smaller objective lenses of about 10–20× magnification to allow a total magnification of 100–200×. An inverted microscope is used to study samples from below. The main advantage is that the objective lens can reach much closer to the specimen, provided that the bottom of the counting device has the thickness of a cover glass, i.e. 100–200 µm. This setup allows the use of 60× objective lenses, which

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
DOI: 10.1079/9781786391759.0003

in combination with 10× eyepiece lenses, will result in overall 600× magnification. The stereo microscope is a low-powered microscope which gives a stereoscopic view of the specimen. Standard stereo microscopes are equipped with objective lenses up to 6× magnification and eyepiece lenses of 10× magnification to give a total magnification of 60×.

Different stains can facilitate the detection and thus the enumeration of nematodes. Possible applications are the counting of nematodes within plant tissues, in dirty liquids or selective counting of living individuals following application of viable stains (see Perry, Chapter 9, this volume). Optical filters for light absorbance, transmission and reflection can improve the contrast and ease of counting. More recently, new developments in image analysis techniques, machine learning and the use of artificial intelligence moved nematode counting away from optical detection by individuals to automatized phenotyping of nematode populations. In addition, molecular tools for quantifying nematodes are becoming increasingly popular in routine diagnosis.

3.2 Estimating Nematodes in Plant Tissue

Reasons for estimating nematode numbers in plant tissues are: (i) to clarify the host status of a plant; (ii) to study the life cycle of endoparasitic nematodes; (iii) to investigate plant–nematode interactions; (iv) to evaluate the effect of plant protection measures on nematode penetration and/or damage; and (v) to analyse the impact of plant defence mechanisms. Nematodes can either be stained in the plant tissue and counted directly within the plant tissue, or after maceration of the plant tissue in a counting slide, or nematodes are first extracted from the plant tissue by the mistifier method (EPPO, 2013) and counted afterwards in liquid (see below). Where plants have been grown in field soil, different plant-parasitic nematode species can coexist within the same root. In those cases, evaluation by direct staining might not be possible due to the loss of morphological features during the staining and heating procedure.

This section describes the enumeration of nematodes within plant tissue (e.g. roots, stems, leaves, seeds). Since nematodes are transparent, they first need to be stained to be visible against the background of the plant tissue. This usually requires a two-step procedure, first destaining of the plant tissue and second, selective staining of the nematodes within the plant tissue. The only exception are *Arabidopsis* roots that are already transparent and thus do not require destaining. Staining of nematodes in bulk material such as old or thick roots or seeds generally requires cutting of the material in thin slices which could be done by hand or with the help of a microtome (Hooper, 1986; Ravichandra, 2010).

3.2.1 Staining nematodes inside plant tissues with acid fuchsin

Nematodes within plant tissue can be stained without staining the associated plant tissue. One of the most widely used stains for nematodes is acid fuchsin (modified after Byrd *et al.*, 1983). This method requires the following materials:

- inverted or stereo microscope
- chlorine solution (e.g. household bleach containing NaOCl)
- acid fuchsin (hazard!) stock solution: 3.5 g acid fuchsin + 250 ml acetic acid + 250 ml distilled water
- sieve of approximately 100 µm aperture to hold the plant sample during various washing steps
- glycerin
- glass vials
- clean water.

Staining of nematodes within plant tissue requires two steps: destaining of the plant tissue and then staining of the nematodes within the plant tissue:

- Wash the plant material free from soil and debris.
- Soak the plant tissue in a 1.5% chlorine solution for about 4 min; the right strength and incubation time of the bleach depends on the plant tissue. Herbaceous material usually clears faster than woody material.

- Thoroughly rinse the plant material in running tap water (30–45 s) by holding the plant material into the water jet or placing it on a sieve to remove all traces of the bleach, which otherwise will inhibit the staining by acid fuchsin.
- Transfer the plant material in a glass vial.
- Cover the plant material with a 1:20 to 1:40 dilution of the acid fuchsin stock solution. The glass vial should not be filled more than half to avoid boiling over during heating.
- Boil the solution in a microwave oven for few seconds in case of young tissue or up to 30 s for older tissue.
- Allow the plant tissue to cool in the staining solution; staining intensity often improves within the first 24 h of storage.

Samples can be evaluated immediately or stored in the staining solution for several days. Before evaluation, place the plant material on a 100 µm sieve and wash off excess stain in running tap water. If discolouration is not sufficient, place the plant tissue in equal volumes of glycerin and water acidified with few drops of lactic acid or 5 N HCl. Finally, place the plant material on a microscope slide in a few drops of glycerin and add a cover glass. The nicely red-stained nematode stages can now be counted at 40–60× magnification under a compound or stereo microscope (Fig. 3.1). Thicker plant material can be placed between two microscope slides that are pressed slightly together to flatten the plant material, which will facilitate counting. For larger samples use a Petri dish cover, add the plant material in a small amount of glycerin and press the bottom of the Petri dish against it. A marked grid on the Petri dish cover will aid in counting the nematodes.

Alternatively, macerate the plant material in a blender (e.g. Waring blender or kitchen blender). The required time and speed of the blender and number of intervals between cycles varies between different plant materials and should be checked beforehand. For example, two intervals of 10 s each at full speed work quite well for 6-week-old tomato roots. The macerate is then transferred in a large (5–10 ml) counting slide. Nematodes will sink to the bottom within few minutes while the plant debris stays on the surface. Nematodes can then be counted under a stereo microscope by focusing on the bottom of the counting slide. To facilitate counting, plant debris can be pushed to the side by using a microscope needle or similar. Alternatively, an inverted microscope could be used and nematodes are counted from below.

Acid fuchsin can be harmful to human health, causing among others skin irritation and eye damage, and thus requires protection gear for handling and disposal of the stain as chemical waste. A safer and easier to handle procedure was described by Thies *et al*. (2002) using red food dye instead of acid fuchsin. A 12.5% solution of McCormick Schilling red food colour (McCormick & Co., Inc., Hunt Valley, MD, USA) containing red 40 (E 129, Allura red) resulted in an excellent contrast of all *Meloidogyne incognita* stages within pepper roots thus providing a safe alternative to acid fuchsin. Further methods are described in Hooper (1986), although some of those methods are no longer in use because of safety reasons, like any staining procedure containing phenol.

Counting stained nematodes in plant tissue is generally more accurate than extracting nematodes with the mistifier technique and counting them in liquid. This is because the mistifier technique does not extract sluggish individuals, sedentary stages or eggs. On the other hand, the staining method also has its limitations.

Fig. 3.1. Different stages of *Meloidogyne incognita* juveniles in tomato roots stained with acid fuchsin. (Photograph courtesy Tim Thoden, JKI Münster.)

It is time consuming, less suitable for older plant material or for very dark plant material. Roots high in phenolics are generally difficult to bleach.

3.3 Estimating Nematodes in Liquids

Extracting nematodes from soil or plants (see Viaene *et al*., Chapter 2, this volume) usually results in a liquid suspension. Depending on the expected density of nematodes in the suspension individuals are counted in aliquots or the entire suspension. Numbers can then be calculated according to the sample size taken. When different taxa are counted, population indices can be computed according to trophic groups or ecological functions.

Enumeration of nematodes in liquids is most likely done by: (i) optical detection using different microscope types; (ii) molecular methods, such as real-time polymerase change reaction (PCR); or (iii) a combination of both. Optical detection methods are attractive by combining them with machine learning techniques and artificial intelligence.

3.3.1 Maintaining a reproducible accuracy in aliquots

Methodological errors in upstream procedures like sampling and extraction can only be identified and determined by evaluating nematode numbers in the final suspension; thus, maintaining a reproducible accuracy of this final step is crucial. Therefore, the dilution factor of the suspension and size of the aliquot have a major impact on the final count. Due to convenience and time efficiency reasons, in most cases nematodes collected from soil or plant samples are counted in an aliquot of, for example, 10 ml out of 100 ml suspension or 1 ml out of 10 ml suspension. The total number is then multiplied by ten to calculate total counts. However, since counting errors decrease with the number of nematodes counted, the greatest accuracy is achieved by counting the entire suspension (McSorley, 1987). This is specifically relevant for nematodes that generally occur at low densities (e.g. *Trichodorus*, *Xiphinema*). For nematodes that occur at higher densities (e.g. *Paratylenchus*, *Pratylenchus*, *Meloidogyne*), counting of 3–5 ml aliquots out of a 10 ml suspension usually gives acceptable accuracy (Seinhorst, 1988). Duplicate counts from the same suspension can help to identify sources of variation but once a certain recurrent variance is achieved by using an established technique it is not necessary to double count in routine samples. Consequently, keeping a target density of about 100 individuals to be counted in a certain aliquot by adjusting the dilution will result in a reproducible counting error of 10% independent of the population density (Southey, 1974). When aliquots are used for counting, care needs to be taken to establish a random distribution of the nematodes. The most commonly used devices to achieve a random dispersion of nematodes are magnetic stirrers, aquarium pumps or accu jets producing air bubbles and Vibro mixers. Once a proper dispersion is achieved, the aliquot should soon be transferred to a counting device and nematodes be counted.

In general, nematode suspensions should be evaluated soon after extraction to avoid losses by microbial contamination or predators co-occurring in those suspensions. If this is not possible, suspensions can be stored at 4–6°C for several days up to weeks. If analysis is envisaged over a longer period, the specimen should be fixed (see Eisenback and Hunt, Chapter 5, this volume).

3.3.2 Using optical methods to estimate numbers in liquids

In most cases, nematodes are manually counted in liquids using a compound, inverted or stereo microscope (Winfield and Southey, 1986). To facilitate nematode counting, various counting devices have been developed (Hooper, 1986; Shepherd, 1986). In principle, they can be divided into rectangular slides or round dishes, which can be open or closed (Fig. 3.2). They come in different sizes depending on the amount of suspension that needs to be counted and the available space under the microscope. In general, counting is done at 40–60× magnification; however, higher magnification (e.g. 200–400×) may be required for small specimens or characters that are not clearly visible at low magnification. For those cases, an inverted microscope is recommended.

Counting nematodes in liquids requires the following materials: compound, inverted or stereo microscope, 100 ml beaker or measuring cylinder made of glass, 10 ml aspirator pipette, aquarium pump or accu jet,

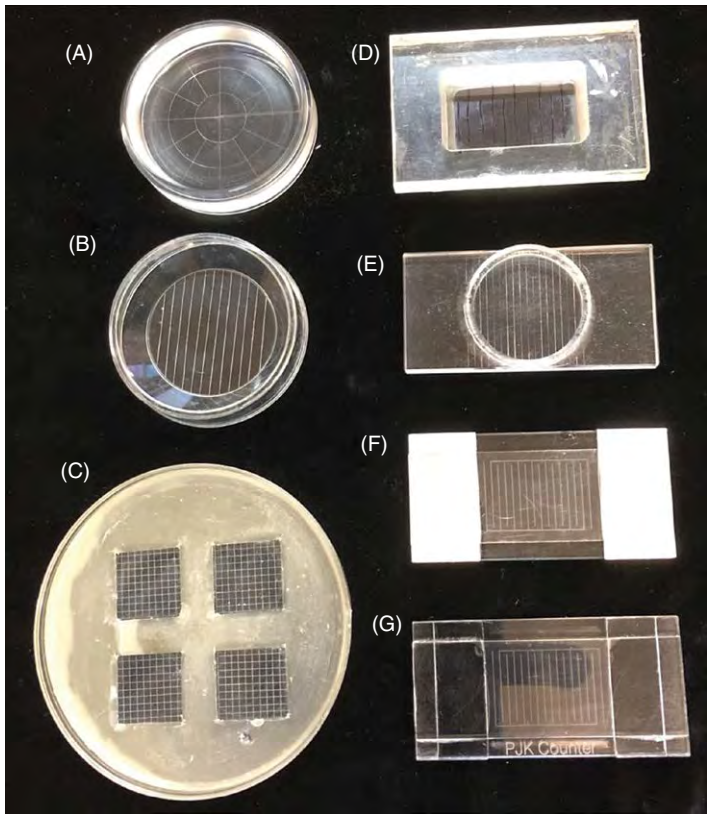

Fig. 3.2. Examples of counting slides/dishes for the enumeration of nematodes or eggs. **(A)** 50 mm diam. counting dish made of glass. **(B)** 55 mm diam. plastic Petri dish with manually cut lines using a plastic or glass writing knife. **(C)** Multichamber counting slide with sloping sides made in paraffin within a 90 mm diam. plastic Petri dish. **(D)** 2 ml counting slide in acrylic glass frame with a 78 × 48 mm cover glass at bottom to allow examination with an inverted microscope. **(E)** Counting slide with sloping sides consisting of a 2 mm high plastic ring glued on a plexiglass plate of 75 × 37 mm (courtesy of R.A. Sikora, Bonn University, Germany). **(F)** 1 ml counting slide in glass (PJK Feinmechanik, St. Augustin, Germany). **(G)** 1 ml counting slide in plexiglass (PJK Feinmechanik).

siphon with water, counting device and tap water. Prepare a homogeneous suspension, transfer the required amount in a counting device and let the nematodes settle for about 30 s before counting the nematodes under the microscope. If done manually, single or multiple (e.g. for counting different developmental stages of eggs) tally counters are handy in aiding the counting procedure (Fig. 3.3). Two counts should be taken if variance is unknown. Repeated counts of the same suspension should not differ more than 5–15%. If variation is higher and counts of the same suspension do not follow a Poisson distribution, the suspension was not thoroughly mixed, nematodes are conglutinated or there is another problem with the suspension or counting (Moriarty, 1963).

Overall, optical methods are straight forward and allow counting of different taxa at the same time. Depending on nematode numbers, nematode diversity, cleanness of the sample and experience of the operator, the time for counting one sample can range from 5 to over 30 min. Limitations of manual counting are: (i) skilled staff are needed; (ii) the time required for analysis increases with sample size and number of nematodes per sample, which restricts high throughput applications; (iii) individual errors increase with number of evaluators and time elapsed for processing due to lack of concentration; and (iv) samples can only be evaluated once. To overcome those limitations, digital imaging procedures with different degrees of automatization and molecular tools have been developed.

Fig. 3.3. Single and multiple tally counter.

3.3.3 Applying digital image analysis to facilitate optical methods

The first approaches using image analysis for automatic recognition of plant-parasitic nematodes date back to 1988 (Fernández-Valdivia *et al.*, 1988). Been *et al.* (1996) further developed this approach and achieved a high accuracy in counting *Globodera* juveniles from hatch test assays. Counting time was reduced by 80% compared with manual counting. Since this early application, speed and storage capacities of computer systems have increased dramatically and new algorithms including machine learning techniques were introduced to process high-resolution pictures in split seconds. Various systems were developed for fundamental research on *Caenorhabditis elegans* (Xian *et al.*, 2013; Jung *et al.*, 2014; Chen *et al.*, 2018). Several automatic applications have been developed to date, including phenotypic features like number of eggs, number of vermiform stages, as well as size, shape, life span or movement of nematodes in bioassays (Stroustrup *et al.*, 2013; Xian *et al.*, 2013; Jung *et al.*, 2014). Holladay *et al.* (2016) reported image capture and processing techniques for automated counting of two species of entomopathogenic nematodes; this is likely to be suitable for counting other microscopic nematodes. Results gained by automated and manual detection are highly correlated ($R^2 > 0.99$) (Jung *et al.*, 2014).

In principle, a microscope equipped with a digital camera takes images of nematode suspensions that are processed by a specific image analysis software. A standard hardware setup used in automated systems comprises a compound or inverted microscope connected to a high-resolution camera and a stage frame with x-y-z steering holding several object slides or well plates (Fig. 3.4). Steering of the stage frame, microscope and camera is usually linked to a high-performance computer allowing rapid image processing and proper data storage. Image libraries for referencing and management of huge data can be handled as internal solution or networked in external data banks. As a first step, pictures are acquired from the camera and grabbed by software, which is calibrated to the optical setup of the microscope (objective, magnification factor) and the camera (number of pixels). The picture then is optimized for further processing (e.g. white balance, contour balance, contrast, noise) and submitted to an image analysis software. The software applies specific algorithms depending on sample type, sample quality, targeted object and unavoidable artefacts. In quantitative assays the objects first need to be identified. This is part of a segmentation process where foreground (target object) and background needs to be distinguished and separated. A typical image segmentation method is based on grey thresholds for evenly illuminated images (Been *et al.*, 1996; Xian *et al.*, 2013; Jung *et al.*, 2014; Al-Tam *et al.* 2015). Nowadays, object detection is realized by machine or deep learning applications as part of an artificial intelligence approach. This system requires a huge dataset of certain target features (e.g. shape of different development stages) training for which is provided by manual annotation of objects (e.g. different development stages). These are labelled by qualified persons according to certain defined features (e.g. round, for shape) in thousands of objects like nematodes. Once the algorithm is able to recognize target objects, phenotypic data will be generated from pictures by detecting, counting and measuring these objects. Subsequent statistics provide information on the analysed groups.

Fig. 3.4. Inverted (left) and compound (right) microscope equipped with scanning stage for eight slides, x-y-z-steering and high-resolution camera connected to a PC for automated image analysis of nematode suspensions.

Most of the image analysis applications were developed on relatively clean nematode suspensions from *in vitro* cultures of *C. elegans*. In plant nematology, most suspensions result from soil or plant extractions and contain a considerable amount of debris. In addition, the nematode species of interest might only be present in very low numbers, creating a technical challenge described as a 'rare object detection' problem (Akintayo *et al*., 2018). To overcome those limitations, Akintayo *et al*. (2018) developed a Convolutional Selective Autoencoder (CSAE) that now is able to recognize eggs of *Heterodera glycines* stained with acid fuchsin when being surrounded, or even overlapped, by debris. The CSAE model reconstructs detected eggs while masking all disturbing objects (e.g. debris) before the image data is further processed through a selectivity function. This results in a considerably high detection accuracy ≥ 95%, even in highly cluttered suspensions. Al-Tam *et al*. (2015) followed a different approach. They developed an automatic method to analyse and count root-knot nematodes within a mixed nematode suspension in microscope images. Possible applications are counting root-knot nematode juveniles in resistance assays or pot experiments. The authors succeeded in producing reliable data similar to manually counting but allowed a much higher throughput of samples independent of operator failures. This method is even able to count the number of nematodes per volume unit and to assess the size of each nematode providing information about different nematode stages or taxa. Most of the image analysis tools described here are available as open source software. They were originally developed for specific environments and applications and need to be adapted for counting nematodes (Moore *et al*., 2013).

3.3.4 Counting nematodes using light absorbance

Nematodes absorb light of a specific wavelength which can be measured by spectrophotometry. Robinson *et al*. (1992) tested the feasibility of counting plant-parasitic nematodes in an aqueous suspension by measuring the light transmittance at 550 nm with an ELISA microplate reader. Absorbance readings correlated well with nematode numbers. The method was successfully tested for eggs of *Meloidogyne incognita*, fourth-stage juveniles of *Ditylenchus phyllobius* and mixed vermiform stages of *Rotylenchulus reniformis*. According to the authors, the method is as accurate as direct counting but more than 100 times faster. Unfortunately, only suspensions of between 2000 and 10,000 eggs or vermiform stages per ml were tested and no information

was provided for accuracy of counting lower densities, which might be more realistic in practice. As pointed out by the authors, the suspension must be very clean as soil particles and debris in the suspension might interfere with the reading.

3.3.5 Counting nematodes using quantitative PCR

Quantitative polymerase chain reaction (qPCR) or real-time PCR allows sensitive detection and species-specific identification combined with quantification of nematode target species. High sensitivity and specificity against a large range of populations of the target species are prerequisites for standard use as a quantification tool. qPCR methods have been developed for the main plant-parasitic nematodes (Braun-Kiewnick and Kiewnick, 2018). qPCR is used in some diagnostics laboratories for routine identification and quantification.

DNA extraction using lysis buffers containing proteinase K and β-mercaptoethanol renders clean DNA for qPCR without further purification or PCR inhibitor removal step (Braun-Kiewnick and Kiewnick, 2018). In most cases DNA is extracted from nematode suspensions. If DNA is extracted directly from soil, prior air-drying of the soil results in more homogenized and representative results (Min *et al*., 2011). Air-drying will also conserve nematode DNA in soil. For example, 5 months storage of air-dried soil at room temperature did not change cycle threshold (Ct) values for *Pratylenchus penetrans* (Sato *et al*., 2010). However, it should be acknowledged that DNA of dead nematodes can persist in soil for at least 6 days (MacMillan *et al*., 2006) and might cause an overestimation if only live nematodes need to be counted.

To enable quantification, development of a standard curve is required. A standard curve is created by plotting Ct values against log-transformed concentrations of serial dilutions of target nematodes. An ideal standard curve, although difficult to achieve, shows highly significant linearity between Ct values and the dilution rates and has an R^2 value of > 0.99 and a slope of –3.32. Most qPCR methods were developed for a single species or a mixture of a limited number of species. If multiple species are targeted (e.g. diagnostic services for farmers), each species requires a standard curve for quantification.

Both underestimation and overestimation of target nematodes with qPCR quantification have been reported (Berry *et al*., 2008; Oliveira *et al*., 2017) and proper validation of the method used is recommended before standard use. Toyota *et al.* (2008) artificially inoculated soil with different numbers of *Globodera rostochiensis* juveniles and, following nematode extraction, obtained a highly significant correlation between numbers of inoculated *G. rostochiensis* juveniles and qPCR values. Sato *et al*. (2011) received higher detection levels and higher numbers of *Pratylenchus* with a combination method of soil compaction and qPCR in comparison to the Baermann method. New approaches aim at the quantitative detection of plant-parasitic nematodes directly from the soil. Min *et al*. (2011) inoculated 20 g of sandy soil with different numbers of *M. incognita* juveniles. The soil was then ground with a mortar and pestle or a ball mill and 0.5 g of the soil was used for DNA extraction. Numbers of *M. incognita* were 15 times higher with the qPCR method than the Baermann method. Although those results are promising, broad practical application will require processing of larger soil samples of 100 ml minimum to consider the heterogeneous distribution of plant-parasitic nematodes in the field and to reflect different soil and environmental conditions. One option could be the combined application of laundry detergent for nematode lysis, Fe_3O_4 super paramagnetic iron oxide nanoparticle (SPION) to capture the DNA, and polyvinylpolypyrrolidone (PVPP) purification (Gorny *et al*., 2018). With this SPION capture method high quality DNA could be extracted from 100 g soil. However, DNA quantities were significantly lower than for standard phenol extraction methods or commercial kits using 0.5 g of soil. Since developments are on-going, robust and reliable qPCR protocols for estimating nematode numbers are expected for the near future.

3.4 Estimating Numbers of Cysts

Cyst number and cyst content are common parameters to describe nematode infestation. Most damage threshold models nowadays refer to cyst content, i.e. the number of eggs and juveniles per 100 ml soil. However, number of cysts can also provide relevant information, such as the history of a field infestation and the age of a cyst. For example, a high number of cysts with low cyst content indicate a severe but old infestation.

Furthermore, cyst numbers per plant might also be a reliable parameter for measuring resistance in high throughput screening protocols, provided that resistant genotypes do not produce empty cysts.

As a result of the specific extraction methods (see Viaene *et al*., Chapter 2, this volume), cysts are collected along with remaining organic float. The amount of organic float depends on soil type, organic matter content and extraction method used. Since the float is rarely clean enough to allow direct counting of the cysts, additional cleaning steps are required.

3.4.1 Using extraction techniques with high efficiency to separate organic debris

Lamondia and Brodie (1987) described an extraction method specifically for organic soils using an underflow elutriator originally designed for extraction of *Striga asiatica* seeds from soil. For *Globodera* and *Heterodera* cysts, this method gave a similar extraction efficiency to the Fenwick can, but organic float was reduced by 50%. Another extraction method for dry or wet samples with very effective separation of the organic float is the modified centrifugal flotation method (Dunn, 1969). This method makes use of the different specific density of cysts and the remains of the float:

- Disperse the float in 300 ml of water.
- Add about 10 g of kaolin.
- Thoroughly mix the suspension with a Vibro mixer for 15 s.
- Centrifuge the suspension for 3 min at 1800 *g*; cysts with a specific density above 1 will precipitate in the kaolin sediment.
- Pass the supernatant through a 125 µm sieve to collect only partially filled or empty cysts.
- Add $MgSO_4$ with a specific density of 1.28 to the sediment.
- Thoroughly mix the suspension with a Vibro mixer for 15 s. In the case of heavy soils forming a solid pellet, a kitchen mixer at slow speed helps to dissolve the pellet.
- Centrifuge the suspension again for 3 min at 1800 *g*.
- Pass the suspension through the 125 µm sieve.
- Rinse the cysts on the sieve with water.
- Collect the sieve in a funnel with filter paper.
- Count the cysts on the filter paper or after transfer in a counting dish.

Species-specific differences occur in floating traits of cysts. For example, cysts of *H. goettingiana* tend to sink to the bottom. Similarly, young cysts of *H. avenae* do not float well. The same applies to older cysts. When cysts do not float sufficiently or cannot be dried because the live cyst content is needed, the entire float has to be investigated. Depending on the proportion and composition of organic debris (e.g. seeds) and experience of the evaluator, hand picking of cysts may take up to 30–45 min per sample and therefore is an unavoidable bottleneck in terms of time.

3.4.2 Using organic solvents to separate cysts from organic float

The easiest way is to dry the float and then separate the cysts from the float (see below). However, rapid drying will kill most species of cyst nematodes (e.g. *Heterodera* spp.), while this method is only suitable if live eggs and juveniles are not required or for species that can tolerate the drying process (e.g. *Globodera* spp.). Cysts are then separated from the dried float by using organic solvents (e.g. ethanol, acetone). This method makes use of the difference in surface structure between cysts and organic debris. The lower surface tension of cysts compared with organic matter will keep them afloat, while the porous organic matter adsorbs the organic solvent and sinks.

The float is transferred into a 250 ml Erlenmeyer flask. The flask is filled to about 75% full with 96% ethanol and the suspension is stirred well. The flask is then filled with 96% ethanol to the top of the neck. When all cysts have floated to the neck of the flask (1–3 min), the floating material is poured, while slowly rotating the flask, onto filter paper in a funnel placed in a second flask. Care is needed to transfer all cysts. Pour no more than half the content of the flask to avoid debris contaminating the sample. Transfer the filter paper containing the cysts in a glass Petri dish for further counting and collecting the cysts under a stereo

microscope. There are several adaptations of this method. Seinhorst (1974) used a supply vessel with ethanol that is connected with a flexible tube to an Erlenmeyer flask containing the cysts. By lifting the supply vessel up, the ethanol flows from the vessel into the flask carrying the cysts to the top of the flasks where they fall over the rim and are collected on a filter. Another modification is replacing the ethanol with acetone (Cantosaenz and Gonzalez, 1993). Adequate safety precautions need to be taken as all those solvents are volatile and highly flammable.

3.4.3 Mechanical separation of cysts (*Globodera* spp.) from organic float

An alternative cleaning step avoiding any use of solvents or additional treatment is possible for *Globodera* spp. This method makes use of the almost round shape of the cysts that roll easily. The float with the dried cysts is passed through an 840 µm sieve over a 250 µm sieve (Shepherd, 1986). The content of the 250 µm sieve is then sprinkled near the top of a smooth board (i.e. made of metal or plastic) about 20 × 30 cm in size. If the board is then held slightly obliquely, cysts will roll down the slope and can be collected. Gentle tapping can facilitate the process. One has to be aware that other round objects such as seeds or mycorrhiza spores will also be collected. An automated version of this method was developed by MEKU - Erich Pollähne GmbH (Wennigsen, Germany) (Fig. 3.5) making use of two vibrating boards having a roughened surface that can be adjusted to an angle of 20°. The dried float moves upwards in opposite direction to the slope, while round cysts roll downwards onto another board. Cysts are finally collected in a small tray, which is placed under a vibrating sieve. Vibration can be controlled independently in both boards. Cysts within the tray can then be counted under a stereo microscope.

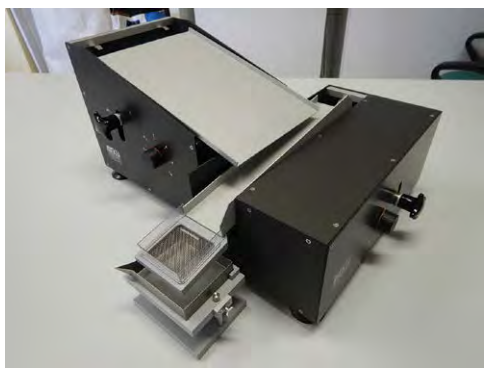

Fig. 3.5. Meku-Extractor for separation of cysts of *Globodera* spp. from organic float in dried samples. Two boards with roughened surface independently can be adjusted to an angle of 20° as well as intensity of vibration; round cysts roll down each board and dried float moves upwards where it can be collected in a chamber at the back of the device.

3.4.4 Direct visual enumeration of cysts attached to roots using transparent boxes

Estimation of cyst numbers attached to roots is conducted in high throughput screening protocols for identification of resistant genotypes or in biotest protocols using transparent pots or transparent folded boxes (Fig. 3.6). These containers are filled with < 300 ml of substrate and can be arranged within very small spaces. Estimating cyst numbers is done after one reproduction cycle at the time of maximum cyst formation. Roots at the transparent surface of the boxes can be directly observed for attached cysts under a stereo microscope. A limitation of this method is the heterogeneous distribution of roots at the transparent surface. This limitation can be partly overcome during plant growth by inclining the boxes at 30° to force 65–80% of the roots to grow to the bottom along the transparent wall of the boxes. It is necessary to keep the transparent wall dark during plant growth and carefully apply fertilizer and water to avoid growth of algae.

3.4.5 Counting females using fluorescence

Brown *et al.* (2010) developed an automated method using fluorescence to count females of the soybean cyst nematode *Heterodera glycines*. Females were washed at 30 days after inoculation into small Petri dishes.

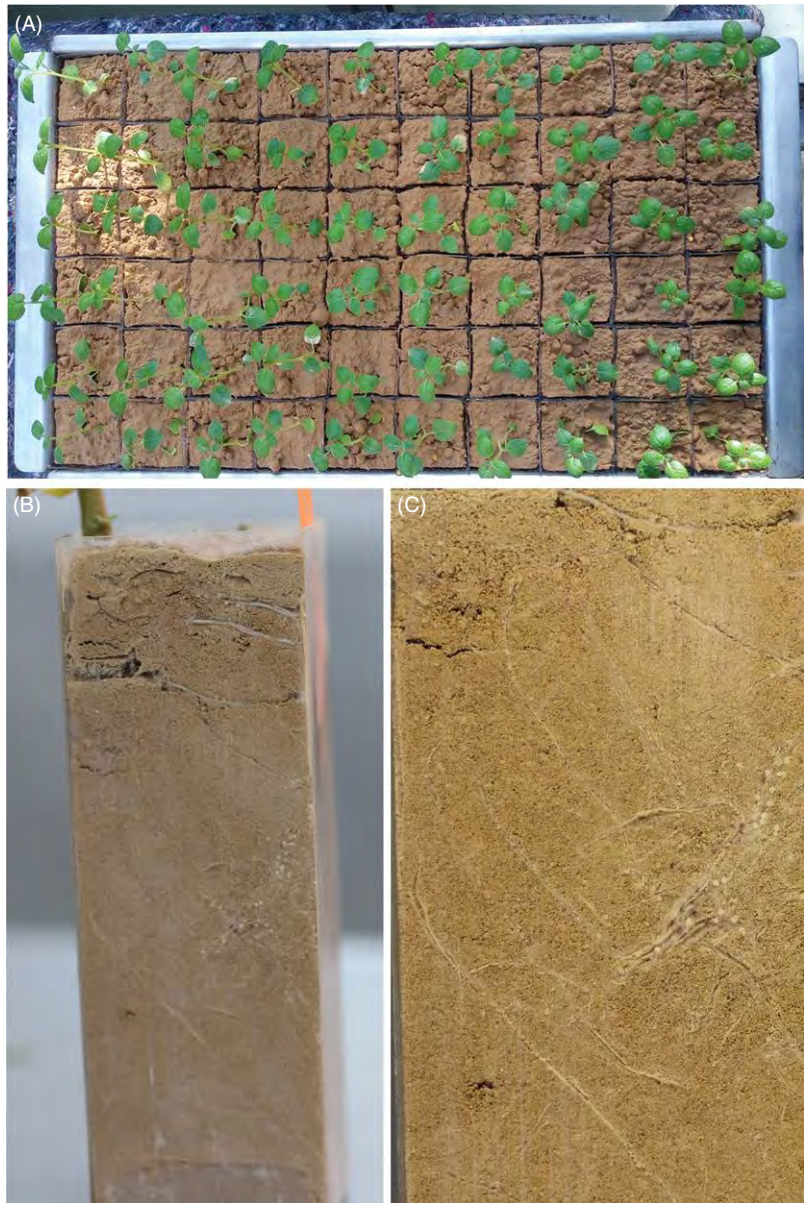

Fig. 3.6. Transparent folded boxes (40 × 40 × 120 mm) for resistance screening of potato plants against *Globodera* spp. **(A)** Sixty plants fit into a metal box. **(B)** Potato roots exposed to the surface of the transparent wall. **(C)** *Globodera* spp. females attached to roots are visible through the transparent wall. (Courtesy of James Mwangi, JKI Braunschweig, Germany.)

The Petri dishes were then scanned with a Kodak Image Station 4000MM Pro using excitation and emission wavelengths of 470 nm and 535 nm, respectively. Fluorescent females were counted with Carestream Molecular Imaging Software. According to the authors the system is as accurate and 50% faster than manual counting under a microscope.

3.5 Estimating Eggs and Juveniles Isolated from Cysts

Once cysts have been extracted and counted, cyst content, i.e. number of eggs and juveniles, can be determined. Cysts can either be crushed by various means (Shepherd, 1986) and the resulting suspension counted as described above (Section 3.3) or cysts can be placed in hatching solution (host diffusate or water, depending on the species) and the hatched juveniles are counted.

3.5.1 Estimating content of cysts using a homogenizer

This method is commonly used to determine the infestation level of a certain cyst nematode species in the field. All cysts from a certain amount or volume of soil are collected in a small Petri dish or glass staining block with a few millilitres of water. Subsequently, cysts are crushed, such as by using a modified homogenizer (Fig. 3.7). The homogenizer consists of an overhead stirrer equipped with rotor and a plastic tube showing a clearance of 1.5–3 mm between wall of the tube and the rotor. The surface of the rotor is provided with a micro channel (300 µm wide, 100 µm deep) that runs helically from the bottom to the top allowing water to float alongside the rotor without destroying juveniles and eggs when cysts are crushed. Collected cysts, together with < 10 ml of water, are placed in the bottom of the tube. The rotor is then inserted into the tube and the tube is gently moved up and down for 1 min at 600–700 rpm. The suspension is then diluted in 20–100 ml depending on required nematode numbers in 1 ml aliquots for counting. Estimation follows the procedure as described in Section 3.3, above. To avoid squashing of juveniles and eggs, it is crucial that users are trained not to apply excessive pressure, which would result in juveniles splitting, and the presence of severed anterior and posterior body parts compromising accurate counting. If the diameter of the rotor is too small (< 3 cm) power is concentrated to a smaller surface and therefore may also result in squashed juveniles.

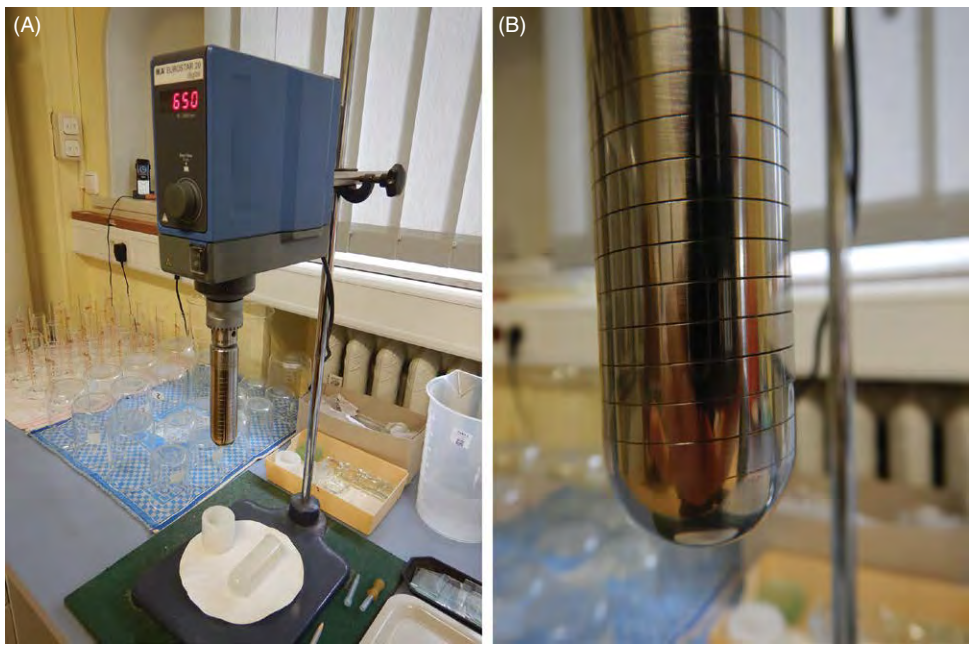

Fig. 3.7. Cyst homogenizer consisting of an overhead stirrer equipped with rotor **(A)** provided with a micro channel (300 µm wide, 100 µm deep), which runs helically from bottom to top. **(B)** Cysts are placed into a plastic tube allowing clearance of 1.5–3 mm between wall of the tube and rotor.

3.5.2 Estimating content of individual cysts by preparing a squash mount

Individual cysts are investigated as a rapid method to get quick information on the status of a population (e.g. maturation, constitution, viability) or on morphological features of cysts and juveniles leading to diagnostic properties of taxonomic groups.

Individual cysts are crushed by hand and a squash mount is prepared. Cysts are placed in a drop of water on top of an object slide for microscopes using flexible forceps or a fine paint brush. A second object slide is placed on top for cover and slightly moved back and forth until cysts burst open and release eggs and juveniles. If necessary, small quantities of water can be added or aspired by inserting a pipette head in the narrow junction between the object slides. Object slides finally can be observed under a compound or inverted microscope and number of eggs and juveniles counted.

3.6 Estimating Gall Index

Several plant-parasitic nematodes cause root galling including *Hemicycliophora*, *Longidorus*, *Xiphinema*, *Nacobbus* and, most importantly, *Meloidogyne*. Galls are induced by secretions of the nematode at the initiation of a feeding site and, therefore, are an indicator of successful nematode establishment within the plant. As a result of nematode secretions, within a few days the root tissue surrounding the nematode undergoes hyperplasia and hypertrophy to form the gall. The number and size of galls depends on nematode species and plant species, but in all cases is positively correlated with nematode infestation. Thus, estimating galling severity is an important parameter to evaluate nematode infestation, especially in the case of *Meloidogyne*. Since galls are clearly visible 1–2 weeks after nematode infection, estimating galling is commonly used as an early parameter for successful nematode establishment in screening programmes for plant resistance or control agents towards *Meloidogyne*. However, it should be kept in mind that estimating the gall index is not a measure for nematode reproduction, which requires counting the next generation. Depending on the plant defence mechanisms, a feeding site can be initiated and a gall formed, but further development of the nematode might be inhibited and reproduction may not occur.

The easiest way to estimate galling is to count the number of galls, either directly or under a stereo microscope, magnifying glass or loupe at 10–20× magnification. However, counting the number of galls has some limitations. First, it is time consuming; second, in addition to the number of galls, the size of galls matters when it comes to plant damage. The earlier the infestation, the larger the galls being formed, and the larger the galls, the more females have penetrated initially (Dropkin, 1954). Thus, gall size can be more relevant than gall number. Finally, the number of galls can be too numerous to be counted, such as when older plants in pot or field experiments have to be evaluated.

To compensate for those limitations, Feldmesser and Feder (1955) developed a gall index for *Meloidogyne* infestation that takes into account the number and size of galls. Based on visual inspection they suggested a rating scheme with values ranging from 0 (no galling) to 4 (heavy galling). According to Zeck (1971), this system did not allow sufficient differentiation between treatments, so he recommended a 0–10 gall index. Bridge and Page (1980) supported such a 0–10 gall index and provided clear definitions for each score (Fig. 3.8).

Today, estimating the gall index is a standard method to evaluate *Meloidogyne* infestation in screening programmes for nematicide efficacy or plant resistance, but it is also successfully used in many other applications. The method is quick, reliable and can easily be adapted to different scenarios, such as *Meloidogyne* species forming small galls (e.g. *M. hapla*, *M. chitwoodi*) versus species forming large galls (e.g. *M. incognita*, *M. enterolobii*) or plants showing small galls (e.g. grasses) versus those showing large galls (e.g. tomato, lettuce). Coyne *et al.* (2014) provided some excellent pictorial score sheets for *Meloidogyne* infestation of lettuce, carrot and cassava. Finally, we should keep in mind that the rating itself is an individual decision and that different examiners might come to different conclusions. Therefore, one experiment should best be evaluated by the same person. If this is not possible, representative root systems can be selected at start of the evaluation to serve all evaluators as reference throughout the evaluation procedure.

There are several modifications to the gall index. Hussey and Janssen (2002) used an index of 0 to 5, where 0 = no galling; 1 = few small galls; 2 = ≤ 25% roots galled; 3 = 26–50%; 4 = 51–75%; and 5 = above 75% roots galled. Taylor and Sasser (1978) also used a 0 to 5 index, but they first counted the number of galls

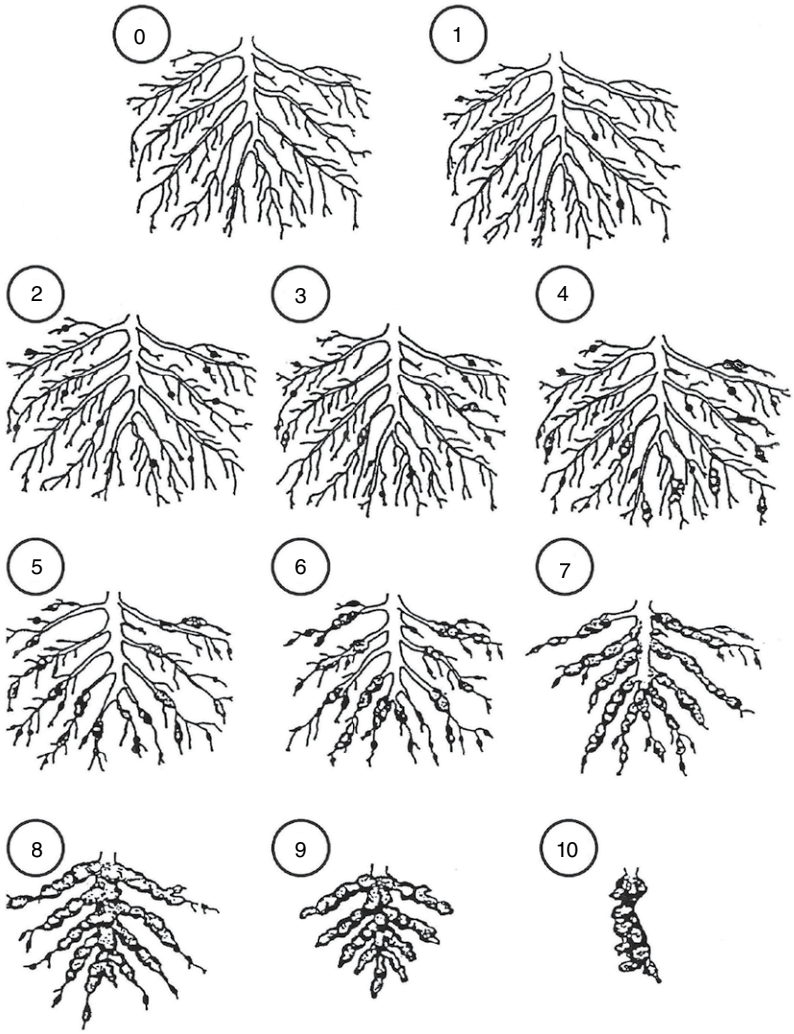

Fig. 3.8. Scheme of a 0–10 index to evaluate root-knot nematode infestation, where 0 = no galling; 1 = few small galls, difficult to find; 2 = small galls only, but clearly visible, main roots clean; 3 = some lager galls visible, main roots clean; 4 = larger galls predominate, but main roots clean; 5 = 50% of roots affected, galls on some main roots, reduced root system; 6 = galls on main root; 7 = majority of main root galled; 8 = all main roots plus tap root galled, few clean roots visible; 9 = all roots severely galled, plant usually dying; 10 = all roots severely galled, no root system, plant usually dead. (Adapted from Bridge and Page, 1980.)

and then assigned them to the different index levels: 0 = no galls; 1 = 1–2; 2 = 3–10; 3 = 11–30; 4 = 31–100; and 5 = above 100 galls. Although the latter index is commonly used, the benefit of first counting the number of galls and then assigning them to a score compared to just counting the number of galls is not always clear. One benefit could be that gall numbers above 100 no longer need to be counted and thus time is saved. Dong *et al*. (2007) compared the above-described two 0–5 gall indices with counting the number of galls and concluded that the gall index as defined by Hussey and Janssen (2002) was more robust in separating peanut genotypes according to their resistance against *M. arenaria* than the other two methods.

3.7 Estimating Number of Egg Masses

Females of several sedentary gall-forming species of nematodes lay their eggs in a gelatinous matrix outside their body to form a so-called egg mass. The gelatinous matrix consists of glycoproteins that are secreted by the female through the anus immediately before and during the egg laying process (Sharon and Spiegel, 1993). It is assumed that if there is an egg mass, it contains eggs, which in most cases is correct. Therefore, counting egg masses is a quick and widely used method to determine nematode reproduction. However, exceptions are possible. As shown for some resistance fodder radish genotypes, a few females were still able to secrete the gelatinous matrix, but did not lay eggs (J. Hallmann, Münster, 2019, personal communication). This can be the case when the resistance mechanisms (e.g. restricted growth of the feeding site) become effective at a very late stage. Counting those 'empty' egg masses will lead to false conclusions. Although this might be an exception, care should be taken in screening procedures relying on egg mass counts.

Most egg masses are transparent or opaque, while others turn brown with maturity. In both cases, they are difficult to see against the white to brownish background of the root surface. To aid counting, egg masses are generally stained beforehand.

3.7.1 Staining egg masses with red food colour

The procedure for staining egg masses with red food stain is as follows:

- Root systems are carefully washed free of adhering soil in a bucket of water or mild stream of tap water; do not use a strong jet of water, which might detach the egg masses.
- Washed root systems are carefully blotted dry on paper tissue or a towel.
- Roots are then soaked in a 500 ml glass beaker containing 1% (w/v) of the red food colour carmine (cochineal red; natural red 4, E 120) or its synthetic versions cochineal red A (E 124 = ponceau 4R; not registered in all countries) or red 40 (E 129, allura red); carmine stains the gelatinous matrix of the egg masses but not the root (Fig. 3.9).
- After 15 min, the roots are removed from the staining solution and carefully rinsed in tap water to remove excessive stain.
- Stained egg masses are finally counted directly or under a stereo microscope, magnifying glass or loupe at 10–20× magnification.

Fig. 3.9. Egg masses of *Meloidogyne hapla* on fodder radish stained with 1% cochineal red A (Brauns-Heitmann, Warburg, Germany).

In the original description, Thies *et al.* (2002) used a 10% or 20% (v/v) solution of McCormick Schilling red food colour (see Section 3.2.1). Damasceno *et al.* (2016) achieved excellent staining and good contrast of egg masses already with a 1% mix of ponceau 4R/red 40 (E 124/E 129) or ponceau 4R/brilliant blue (E 124/E 133), respectively. In principle, the optimum concentration might vary depending on nematode species, plant species, age of the root system and source of the stain. Therefore, it is best for users to define the optimum conditions for their purpose in preliminary tests. In the past, phloxine B was commonly used (Holbrook *et al.*, 1983). Phloxine B also stains the egg masses red just like carmine, but its use is more stringent in requiring personal safety protective equipment and waste disposal, which hinders routine application.

3.7.2 Estimating egg numbers isolated from egg masses

Most sedentary plant-parasitic nematodes lay their eggs agglomerated within an egg mass or cyst. As for counting cysts (see Section 3.4), counting the number of egg masses can be unsatisfactory as the content of egg masses varies greatly depending on nematode species, developmental stage of the female and host plant. To estimate the number of eggs, individual egg masses can be picked, placed in a drop of water on a microscope slide and after adding slight pressure to separate the eggs within the egg mass, eggs can be counted under a compound microscope at 40–60× magnification. However, this procedure is tedious, time consuming, and only works for small numbers.

For a more reliable count, eggs first have to be released from the gelatinous matrix of the egg masses. For egg masses, chlorine is used to destroy the gelatinous matrix (Hussey and Barker, 1973; Veremis and Roberts, 1996). A common protocol is as follows:

- Shake the roots with egg masses for 5 min in 1% NaOCl.
- Rinse the suspension over nested sieves of 250 µm and 20 µm.
- Finally, wash thoroughly with tap water.
- Discard the root debris collected on the 250 µm sieve.
- Transfer the eggs on the 20 µm sieve into a glass beaker.
- Count either the entire suspension, or aliquots of it, under the compound or inverted microscope at 40–60× magnification. Counting can be done either manually or by digital analysis (see also Section 3.3).

To facilitate counting, Byrd *et al.* (1972) stained the eggs by adding two drops of a 0.35% acid fuchsin solution in 25% lactic acid to the egg suspension, which was then boiled for 1 min. As already mentioned in Section 3.2.1, handling with acid fuchsin requires wearing protective gear and appropriate waste disposal. To avoid this, various food dyes can be used instead. For example, a 4% mix of ponceau 4R/red 40 (E 124/E 129) or ponceau 4R/brilliant blue (E 124/E 133) will be an efficient substitute (Damasceno *et al.*, 2016). Depending on plant material and plant age, the concentration and incubation times of the dye needs to be adapted.

Several modifications of the common protocol are used in practice. Depending on nematode species, plant species and plant age, parameters such as NaOCl concentration, extraction time and sieve size can be adapted. It is recommended to define the optimum conditions in preliminary tests.

In principle, either a defined number of selected egg masses can be treated, but most commonly, entire root systems are processed. Using only parts of the root system is not recommended due to the heterogeneous distribution of egg masses over the root system. In case of large galls, such as for *M. incognita* or *M. enterolobii* on tomato, significant numbers of eggs are laid within the root tissue making their recovery difficult. In those cases, the root tissue is macerated in 1% NaOCl in a blender for 30 s (Veremis and Roberts, 1996) and eggs are separated from root debris by sieving. Good results are generally achieved using a 100 µm aperture sieve to retain the root debris and a 20 µm aperture sieve to collect the eggs. Always test the optimum time of maceration and sieve size beforehand. Using a 50 µm sieve instead of a 100 µm sieve to retain root debris will result in a cleaner egg suspension, but several eggs may have been retained on the 50 µm sieve and lost for counting.

3.8 References

Akintayo, A., Tylka, G.L., Singh, A.K., Ganapathysubramanian, B., Singh, A. and Sarkar, S. (2018) A deep learning framework to discern and count microscopic nematode eggs. *Scientific Reports* 8, 9145. DOI: 10.1038/s41598-018-27272-w

Al-Tam, F., dos Anjos, A., Bellafiore, S. and Shahbazkia, H.R. (2015) Detection of root-knot nematodes in microscopy images. *Proceedings of the International Joint Conference on Biomedical Engineering Systems and Technologies Volume 2*. Lisbon, Portugal, January 12-15, 2015, pp. 76–81. DOI: 10.5220/0005209000760081

Been, T.H., Meijer, E.M.J., Beniers, A.E. and Knol, J.W. (1996) Using image analysis for counting larvae of potato cyst nematodes (*Globodera* spp.). *Fundamental and Applied Nematology* 19, 297–304.

Berry, S.D., Fargette, M., Spaull, V.W., Morand, S. and Cadet, P. (2008) Detection and quantification of root-knot nematode (*Meloidogyne javanica*), lesion nematode (*Pratylenchus zeae*) and dagger nematode (*Xiphinema elongatum*) parasites of sugarcane using real-time PCR. *Molecular and Cellular Probes* 22, 168–176. DOI:10.1016/j.mcp.2008.01.003

Braun-Kiewnick, A. and Kiewnick, S. (2018) Real-time PCR, a great tool for fast identification, sensitive detection and quantification of important plant-parasitic nematodes. *European Journal of Plant Pathology* 152, 271–283. DOI: 10.1007/s10658-018-1487-7

Bridge, J. and Page, S.L.J. (1980) Estimation of root-knot nematode infestation levels on roots using a rating chart. *Tropical Pest Management* 26, 296–298. DOI: 10.1080/09670878009414416

Brown, S., Yeckel, G., Heinz, R., Clark, K., Sleper, D. and Mitchum, M.G. (2010) A high-throughput automated technique for counting females of *Heterodera glycines* using a fluorescence-based imaging system. *Journal of Nematology* 42, 201–206.

Byrd, D.W., Ferris, H. and Nusbaum, C.J. (1972) A method for estimating numbers of eggs of *Meloidogyne* spp. in soil. *Journal of Nematology* 4, 266–269.

Byrd Jr, D.W., Kirkpatrick, T. and Barker, K.R. (1983) An improved technique for clearing and staining plant tissues for detection of nematodes. *Journal of Nematology* 15, 142–143.

Cantosaenz, M. and Gonzalez, A. (1993) Use of gasoline to separate organic-matter from cysts of *Globodera pallida* extracted from soil. *Nematropica* 23, 57–61.

Chen, W.Y., Li, W.W., Dong, X.J. and Pei, J.L. (2018) A review of biological image analysis. *Current Bioinformatics* 13, 337–343. DOI: 10.2174/1574893612666170718153316

Coyne, D.L., Nicol, J.M. and Claudius-Cole, B. (2014) *Practical Plant Nematology: A Field and Laboratory Guide. 2nd edn.* International Institute of Tropical Agriculture, Cotonou, Benin.

Damasceno, J.C.A., Soares, A.C.F., de Jesus, F.N. and Castro, J.M. (2016) Root-knot nematode staining with artificial food dyes. *Nematoda* 2016 (3), e182016. DOI: 10.4322/nematode.01816

Dong, W.B., Holbrook, C.C., Timper, P., Brenneman, T.B. and Mullinix, B.G. (2007) Comparison of methods for assessing resistance to *Meloidogyne arenaria* in peanut. *Journal of Nematology* 39, 169–175.

Dropkin, V.H. (1954) Infectivity and gall size in tomato and cucumber seedlings infected with *Meloidogyne incognita* var. *acrita* (root-knot nematode). *Phytopathology* 44, 43–49.

Dunn, R.A. (1969) Extraction of cysts of *Heterodera* species from soils by centrifugation in high density solutions. *Journal of Nematology* 1, 7.

EPPO (2013) PM7/119 Nematode extraction. *Bulletin OEPP/EPPO Bulletin* 43, 471–495. DOI: 10.1111/epp.12077

Feldmesser, J. and Feder, W.A. (1955) Techniques involved in nematocide screening. *Proceedings of the Florida State Horticultural Society* 68, 103–107.

Fernández-Valdivia, J., Castillo, P. and Gómez-Barcina, A. (1988) Use of image analysis for automatic recognition of nematodes. In: Fortuner, R. (ed.). *Nematode Identification and Expert System Technology*. Plenum Press, New York, New York, USA, pp. 293–299. DOI: 10.1007/978-1-4684-9016-9_28

Gorny, A.M., Hay, F.S., Wang, X. and Pethybridge, A.J. (2018) Isolation of nematode DNA from 100 g of soil using Fe_3O_4 super paramagnetic nanoparticles. *Nematology* 20, 271–283. DOI: 10.1163/15685411-00003140

Holbrook, C.C., Knauft, D.A. and Dickson, D.W. (1983) A technique for screening peanut for resistance to *Meloidogyne arenaria*. *Plant Disease* 67, 957–958. DOI: 10.1094/PD-67-957

Holladay, B.H, Willett, D.S. and Stelinski, L.L. (2016) High throughput nematode counting with automated image processing. *BioControl* 61, 177–183. DOI: 10.1007/s10526-015-9703-2

Hooper, D.J. (1986) Preserving and staining nematodes in plant tissues. In: Southey J.F. (ed.) *Laboratory Methods for Work with Plant and Soil Nematodes*. Her Majesty's Stationery Office, London, UK, pp. 81–85.

Hussey, R.A. and Barker, K.R. (1973) A comparison of methods of collecting inocula of *Meloidogyne* spp. including a new technique. *Plant Disease Reporter* 57, 1025–1028.

Hussey, R.S. and Janssen, G.J.W. (2002) Root-knot nematode: *Meloidogyne* species. In: Starr, J.L., Cook, R. and Bridge, J. (eds). *Plant Resistance to Parasitic Nematodes*. CAB International, Wallingford, UK, pp. 43–70. DOI: 10.1079/9780851994666.0001

Jung, S.K., Aleman-Meza, B., Riepe, C. and Zhong, W.W. (2014) QuantWorm: A comprehensive software package for *Caenorhabditis elegans* phenotypic assays. *PLoS One* 9, e84830. DOI: 10.1371/journal.pone.0084830

LaMondia, J.A. and Brodie, B.B. (1987) Extraction of cyst nematodes from organic soils. *Journal of Nematology* 19, 104–107.

MacMillan, K., Blok, V., Young, I., Crawford, J. and Wilson, M.J. (2006) Quantification of the slug parasitic nematode *Phasmarhabditis hermaphrodita* from soil samples using real time qPCR. *International Journal for Parasitology* 36, 1453–1461. DOI: 10.1016/j.ijpara.2006.08.005

McSorley, R. (1987) Extraction of nematodes and sampling methods. In: Brown, R.H. and Kerry, B.R. (eds) *Principles and Practice of Nematode Control in Crops*. Sydney, Australia, Academic Press, pp. 13–41.

Min, Y.Y., Toyota, K., Goto, K., Mizuguchi, S., Abe, N., Nakano, A. and Sawada, E. (2011) Development of a direct quantitative detection method for *Meloidogyne incognita* in sandy soils and its application to sweet potato cultivated fields in Tokushima prefecture, Japan. *Nematology* 13, 95–102. DOI: 10.1163/138855410X504916

Moore, B.T., Jordan J.M. and Baugh L.R. (2013) WormSizer: High-throughput analysis of nematode size and shape. *PLoS One* 8. e57142. DOI: 10.1371/journal.pone.0057142

Moriarty, F. (1963) Variance of counts from larval suspension of eelworms. *Plant Pathology* 12, 177–179.

Oliveira, C.M.G., Blok, V., Neilson, R., Mróz, T. and Roberts, D. (2017) Hydrolysis probe-based PCR for detection of *Pratylenchus crenatus*, *P. neglectus* and *P. penetrans*. *Nematology* 19, 81–91. DOI: 10.1163/15685411-00003033

Ravichandra, N.G. (2010) *Methods and Techniques in Plant Nematology*. New Dehli, India, PHI Learning Private Limited, pp. 595.

Robinson, A.F., Veech, J.A. and Heald, C.M. (1992) Counting nematodes with a microplate reader. *Journal of Nematology* 24, 92–95.

Sato, E., Goto, K., Min, Y.Y., Toyota, K. and Suzuki, C. (2010) Quantitative detection of *Pratylenchus penetrans* from soil by using soil compaction and real-time PCR. *Nematological Research* 40, 1–6. DOI: 10.3725/jjn.40.1

Sato, E., Suga, Y., Kisaki, C., Toyoto, K., Miyake, K., Takada, A., Takeuchi, K. and Matsuura, R. (2011) Quantification of *Pratylenchus penetrans* in radish fields using a combination method of soil compaction and real-time PCR to determine the economic threshold. *Soil Science and Plant Nutrition* 57, 213–220. DOI: 10.1080/00380768.2011.574233

Seinhorst, J.W. (1974) Separation of *Heterodera* cysts from dry organic debris using ethanol. *Nematologica* 20, 367–369.

Seinhorst, J.W. (1988) *The Estimation of Densities of Nematode Populations in Soil and Plants*. Uppsala, Sweden, Växtskyddsrapporter, Jordbruk, pp. 107.

Sharon, E. and Spiegel, Y. (1993) Glycoprotein characterization of the gelatinous matrix in the root-knot nematode *Meloidogyne javanica*. *Journal of Nematology* 25, 585–589.

Shepherd, A.M. (1986) Extraction and estimation of cyst nematodes. In: Southey, J.F. (ed.) *Laboratory Methods for Work with Plant and Soil Nematodes*. Her Majesty's Stationery Office, London, UK, pp. 31–49.

Southey, J.F. (1974) Methods for detection of potato cyst nematodes. *Bulletin OEPP/EPPO Bulletin* 4, 463–473. DOI: 10.1111/j.1365-2338.1974.tb02394.x

Stroustrup, N., Ulmschneider, B.E., Nash, Z.M., Lopez-Moyado, I.F., Apfeld, J. and Fontana, W. (2013) The *Caenorhabditis elegans* lifespan machine. *Nature Methods* 10, 665–670.

Taylor, A.L. and Sasser, J.N. (1978) *Biology, Identification and Control of Root-knot Nematodes (*Meloidogyne *species)*. North Carolina State University and United States Agency for International Development, Raleigh, North Carolina, USA.

Thies, J.A., Merrill, S.B. and Corley, E.L. (2002) Red food coloring stain: New, safer procedures for staining nematodes in roots and egg masses on root surfaces. *Journal of Nematology* 34, 179–181.

Toyota, K., Shirakashi, T., Sato, E., Wada, S. and Min, Y.Y. (2008) Development of a real-time PCR method for the potato-cyst nematode *Globodera rostochiensis* and the root-knot nematode *Meloidogyne incognita*. *Soil Science and Plant Nutrition* 54, 72–76. DOI: 10.1111/j.1474-0765.2007.00212.x

Veremis, J.C. and Roberts, P.A. (1996) Identification of resistance to *Meloidogyne javanica* in the *Lycopersicon peruvianum* complex. *Theoretical and Applied Genetics* 93, 894–901. DOI: 10.1007/BF00224091

Winfield, A.L. and Southey, J.F. (1986) Use of the optical microscope in nematology. In: Southey, J.F. (ed.) *Laboratory Methods for Work with Plant and Soil Nematodes*. Her Majesty's Stationery Office, London, UK, pp. 95–106.

Xian, B., Shen, J., Chen, W.Y. and Han, J.-D.J. (2013) WormFarm: a quantitative control and measurement device toward automated *Caenorhabditis elegans* aging analysis. *Aging Cell* 12, 398–409. DOI: 10.1111/acel.12063

Zeck, W.M. (1971) A rating scheme for field evaluation of root-knot nematode infestations [Ein Bonitierungsschema zur Feldauswertung von Wurzelgallenbefall]. *Pflanzenschutz-Nachrichten Bayer* 24, 144–147.

4 Screening Plants for Resistance/ Susceptibility to Plant-parasitic Nematodes

WIM WESEMAEL

Flanders Research Institute for Agriculture, Fisheries and Food, Merelbeke, Belgium; Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

4.1 Introduction

Plant-parasitic nematodes are less mobile with comparatively low reproductive rates compared, for example, to most insects, allowing clear separation of resistance and tolerance as independent characteristics of the host (Trudgill, 1991). Resistance is defined as inhibition of reproduction. Completely or highly resistant plants allow no nematode reproduction or only trace amounts, respectively. Low to moderate (partial or intermediate) resistant plants support moderate or low multiplication relative to reproduction on a plant lacking such resistance. A susceptible plant allows nematode reproduction. Initial screening for resistance involves evaluating a (large) number of individual plants of a plant species, cultivar or breeding line to identify those on which nematodes cannot reproduce. To allow comparison between years and in independent tests, screening tests should provide consistent and reproducible results. In this chapter, methods for initial screening will be presented that allow relatively fast and high throughput of plants, cultivars or breeding lines. Specific protocols for screening and further resistance breeding on several plant–nematode combinations are described in *Plant Resistance to Parasitic Nematodes* edited by Starr *et al*. (2002). Speijer and De Waele (1997) provide practical guidelines for screening of *Musa* germplasm, and several screening procedures for cyst nematodes are given by Blok *et al*. (2018).

4.2 Selection of Nematode Population

Differences in reproduction between populations of the same nematode species have been reported. These populations are called races when there is a different reaction on hosts from widely different plant families; pathotypes when there are different reactions on hosts, cultivars or breeding lines from the same or related plant species; or biotypes when both are found (Roberts, 2002). Indiscriminate use of these terms for different nematode groups may be confusing. Knowledge on differences between populations is important for resistance screening.

When detection of the highest level of resistance is required, screening should be done with an aggressive (virulent) population (Hussey and Boerma, 1981). Aggressiveness can be evaluated using a known susceptible

 Techniques for Work with Plant and Soil Nematodes, (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
DOI: 10.1079/9781786391759.0004

cultivar from the plant species that will be screened. If such a cultivar is not available, the aggressiveness can be tested on a known host from the same plant family. The aggressiveness should be tested under the same conditions as the resistance screening. To maintain the aggressiveness of the nematode populations during culture it is important that the pure cultures are kept on different host plants in a rotation. It is preferable to test the aggressiveness of stock cultures before a screening assay is initiated.

- A mixture of geographical distinct populations can be used to cover genetic diversity between populations.
- A mixture of aggressive populations originating from distinct geographical regions will identify a broad resistance that can be used in a wider geographical area (Hussey and Boerma, 1981).
- When the geographical area of the crop for which resistance breeding is done is limited, it is preferable to use nematode populations from the target area.
- After initial screening, further screening with a single highly aggressive population allows discrimination of genotypes with the highest level of resistance.

Mixing aggressive populations can exclude sources of resistance that could be combined during further breeding programmes. For potato cyst nematodes, standard populations to be used in resistance assays are given in the EPPO standard PM3/68 (OEPP/EPPO, 2006).

4.3 Collection and Preparation of Inoculum

The use of field soil is undesirable because of non-uniformity of inoculum. A field population may exhibit various levels of dormancy and may be contaminated with parasites and predators. Pure cultures should be established from a few individuals. Eggs, juveniles or adults (gravid females) can be surface sterilized (see Manzanilla-López and Ehlers, Chapter 6, this volume) and the population can be increased for several generations to obtain sufficient numbers for screening tests.

During culture, the purity should be checked regularly. If the nematode species has a multiple host range, a rotation of host plants can be used to maintain the aggressiveness during culture.

4.3.1 Inoculum of cyst nematodes

Cyst nematodes can be inoculated as cysts, eggs or hatched second-stage juveniles (J2). J2 provide greater control over quality and quantity than eggs and cysts. Hatched J2 give precise control over the initial population density (*Pi*). The collection of large quantities of J2 in a short period of time can be difficult. When eggs or cysts are used for inoculation several factors can influence hatching. Under favourable conditions *Heterodera glycines* produces eggs in an egg sac that hatch readily in water, while under unfavourable conditions eggs are retained in the cyst and require host root diffusates for hatching (Ishibashi *et al*., 1973). Similar differences in hatchability of J2 from eggs in an egg sac compared with eggs inside the cyst have been observed for *H. carotae* (Greco, 1981; Aubert, 1986) and *H. goettingiana* (Greco *et al*., 1986). Host plant condition during the growing season can affect hatchability and dependence on root diffusates (Masler and Perry, 2018). Obligate diapause, as observed for *Globodera rostochiensis*, *G. pallida* and *H. avenae*, needs to be considered when cysts are used as inoculum. Diapause can be terminated by exposure to low temperatures during a fixed period (several weeks) and can also be circumvented in cultured cysts by avoiding desiccation (Janssen *et al*., 1987; see Perry, Chapter 9, this volume). When cysts are used as inoculum the mean number of eggs and juveniles in the cysts must be determined and hatchability should be checked. For potato cyst nematodes, inoculum can be accumulated and stored as dried cysts.

4.3.2 Inoculum of root-knot nematodes

Pure cultures of root-knot nematodes, *Meloidogyne* spp., can be obtained from single egg masses. Egg masses, free eggs or hatched J2 can serve as inoculum. Collection of egg masses is time consuming. Roots of infected plants need to be washed carefully to rinse off soil without removing egg masses. Egg masses can be hand-picked with the aid of forceps and should be kept moist. Egg masses differ in size (number of eggs inside)

and may contain eggs in different states of development making proper homogenization and standardization of the inoculum difficult. Inoculation with multiple egg masses can reduce the variation. Depending on the generation or the condition of the host plant hatchability may alter. Number of eggs per egg mass and hatching rate should be checked from a subsample of the egg masses that will be used for the inoculum. Egg masses cannot be dispersed in the soil and may harbour pathogenic microorganisms.

Egg suspensions can be obtained easily and can provide large quantities of standardized inoculum in a relatively short time compared with egg masses. Eggs can be surface sterilized. The suspensions can be distributed uniformly around the roots. To obtain egg suspensions the method described by Hussey and Barker (1973) using sodium hypochlorate (NaOCl), modified by Hussey and Janssen (2002) can be used.

- Wash soil from infected roots > 45 d after inoculation (depending on temperature) when egg masses peak.
- Cut off roots and wash well; use tap water throughout.
- Place roots in a 1 l container and add 200 ml of a 0.525% NaOCl solution (higher concentrations affect viability); close the container and shake vigorously for approximately 3 min, but not longer than 4 min as this will reduce egg viability.
- After shaking, quickly pass the NaOCl solution over a 75 µm pore sieve nested in a 25 µm pore sieve and fill the container with the roots with water. Remove the 75 µm pore sieve and thoroughly rinse the eggs on the 25 µm pore sieve with water to remove residual NaOCl. Rinse the eggs from the 25 µm pore sieve into a beaker containing water.
- Rinse the roots from the first container with water to remove additional eggs and collect them through sieving; do this at least twice.
- Determine the concentration of the egg suspension.

For *Meloidogyne hapla* and *M. incognita*, infectivity of J2 from egg suspensions obtained with NaOCl was higher than J2 collected in a mist chamber but lower than inoculation with intact egg masses (Hussey and Barker, 1973). As with egg masses, hatchability of a subsample of the inoculum should be checked.

J2 as inoculum are more sensitive to handing compared to eggs and egg masses. Infectivity can be lost with storage and it is recommended to use J2 within a short time after hatching (24 to 48 h).

4.3.3 Inoculum of root-lesion and burrowing nematodes

Both *Pratylenchus* spp. and *Radopholus* spp. are widely cultured on carrot discs starting from gravid females. This *in vitro* culture allows mass production of inoculum in a standardized way. Loss of infectivity of *Pratylenchus* spp. and *Radopholus similis* from carrot disc cultures has not been reported (De Waele and Elsen, 2002). To obtain inoculum from carrot discs, the following protocol adapted from De Waele and Elsen (2002) can be used.

- Select carrot disc cultures where many nematodes can be seen on the Petri dish and/or the carrot disc.
- Rinse the Petri dish with distilled water to remove nematodes and pour the water through a 25 µm sieve.
- Rinse the nematodes on the sieve with tap water to eliminate bacteria, etc., and collect the nematodes on the sieve with distilled water in a beaker.
- Collect the nematodes on or in the carrot disc with one of the extraction techniques described in Viaene *et al.*, Chapter 2, this volume.

Mixtures of adults and juveniles are used as inoculum. Inoculum can be stored at cool temperatures, but this can affect the survival and infectivity. Storage of *Pratylenchus penetrans* at 4°C had a negative effect on survival of juveniles from 7 days onwards, whilst adults were not affected over a period of 30 days. Storage at low temperature reduced penetration of juveniles and adults into rose seedlings (Peng and Moens, 1999).

4.3.4 Inoculum of stem and bulb, and foliar nematodes

Ditylenchus dipsaci and *D. destructor* inoculum can be obtained from carrot disc cultures (see Manzanilla-López and Ehlers, Chapter 6, this volume). For *D. dipsaci*, large quantities of inoculum can be reared on alfalfa tissue in a short period of time with the method described by Faulkner *et al.* (1974). Wang *et al.* (2016)

described a technique, the tuber hole technique, to obtain large numbers of *D. destructor* on sweet potato with high efficiency and lack of contamination. Different protocols for monoxenic cultures of *Ditylenchus* spp. have been compiled by Plowright *et al.* (2002). The life cycle of *Aphelenchoides* spp. is short, allowing mass culture in a limited period of time. Monoxenic cultures on carrot discs, clover and alfalfa callus and fungal cultures all allow fairly rapid collection of inoculum of *Aphelenchoides* spp. Macerated infected plant tissues are a source of inoculum that can be obtained easily. Pathogenicity can change depending on the culture method (Zhen *et al.*, 2012). Infectivity or aggressiveness of *Ditylenchus* spp. reared on monoxenic cultures should be monitored on the field host.

After isolation from cultures, inoculum of *Ditylenchus* spp. should be used as soon as possible. Nematodes can be stored in shallow water (5 mm) for 1 to 2 weeks at 2–5°C and maintain good infectivity (Plowright *et al.*, 2002). *Aphelenchoides* spp., such as *A. besseyi* in rice grains in an anhydrobiotic state, can be stored for a longer period before being used as a the source of inoculum.

4.3.5 Inoculum of semi-endoparasitic and ectoparasitic nematodes

Rotylenchulus spp. can be reared on a variety of host plants with a relatively short life cycle under optimal temperatures. Young females are the infective stage, hence the delay between hatching and infectivity. Inoculation can be done with egg suspensions or juveniles can be kept in water until moulting to adult females is completed. This will provide developmentally synchronous but differentially starved nematodes (Balasubramanian and Ramakrishnan, 1983). The culture of *Tylenchulus semipenetrans* is more difficult and requires 6 to 12 months to build up high population densities. Eggs and juveniles can be used as the inoculum.

Ectoparasitic nematodes often have lower fecundity and longer life cycles. Pure cultures can be maintained on host plants and used as a source of inoculum.

4.4 Setting up Resistance Screening Assays

For initial evaluation of resistance levels, screening under controlled conditions is preferred above field screening (Fassuliotis, 1979; Boerma and Hussey, 1992). The latter can be used for advanced generation breeding lines when performance under natural conditions is important. Glasshouses are not optimum as control of lighting period, temperature and relative humidity often fluctuates depending on outdoor conditions. This might influence results and makes it difficult to compare data between years, or data obtained at different periods in the same year. Growth chambers provide a better level of standardized conditions and allow year-round screening.

Nematode development is strongly influenced by temperature. Temperature should be monitored (in the soil if possible). For initial screening, a stable temperature regime (day/night) allowing optimal nematode development and normal plant growth is recommended. Critical steps in nematode parasitism such as attraction, penetration and establishment of a feeding site, can have different optimum temperatures (Bird and Wallace, 1965; Mizukubo and Adachi, 1997; Khan *et al.*, 2014). High temperatures can break the resistance. Photoperiod and radiation should be measured in the 400–700 nm waveband (photosynthetically active radiation); relative humidity (reported as %RH) should be measured near the plant canopy.

A susceptible control, preferably giving high multiplication of the nematodes, should be included. If available, a known resistant control can be included. The number of replicates depends strongly on the genetic variability of the test plant. In general, higher genetic diversity requires a higher number of replicates to reflect the variation in the plant. Cross-pollinators show greater genetic variation than self-pollinators.

4.4.1 Pot type and growing medium

A variety of pot types, containers, tubes, Petri dishes and other devices can be used for resistance screening. In general, the chosen device should allow normal plant development within the time frame of the screening experiment. Differences in size of the root system and plant vigour can have a large effect on nematode

multiplication. To reduce space and quantity of inoculum required, several authors have developed screening protocols using small containers, tubes or Petri dishes.

Small (0.5 l; 9–10 cm diam.), transparent, closed (with lid) plastic containers can be used for screening of potatoes for resistance to *Globodera* spp. rendering reproducible data in a more rapid and economical way compared to pot tests (Phillips *et al*., 1980). They can also be used to screen potatoes for *Meloidogyne* spp. (Wesemael *et al*., 2014). Cyst, galls and egg masses can be seen easily on the roots through the transparent container (Fig. 4.1). Closed containers are kept in the dark and normal aerial plant growth, which could influence nematode development, is blocked.

Petri dishes with water agar or other growing media are also used for screening (Sijmons *et al*., 1991; Blok *et al*., 2018) but have limitations. Sealing of Petri dishes with Parafilm® induces stress to plants and can alter the gene expression profile, plant metabolism and adaptive responses (Xu *et al*., 2019). Roots grown in Petri dishes can easily be exposed to illumination, which induces an immediate and strong burst of reactive oxygen (ROS) production (Yokawa *et al*., 2011). An opening at one side of the Petri dish allows the aerial plant part to grow out of the dish, while the roots in the dish can be kept in the dark. A second opening opposite the first one, allows roots to grow partly out of the Petri dish so they can be submerged in water or a nutrient solution. The advantage of Petri dishes is that the root system can easily be scored for nematode development (galling, egg masses, females, cysts).

Growth pouches (Fig. 4.2) are a good alternative for plants with large seeds such as cowpea (*Vigna unguiculata*) and common bean (*Phaseolus vulgaris*). They allow plant growth with a clean root system that can be easily evaluated. Growth pouches are placed in a plastic hanging folder and arranged in a rack in a vertical position reducing space required (Atamian *et al*., 2012).

Rootrainers™ (available in different sizes) open up like a book (Fig. 4.3) and allow inspection of the root system for nematode development. The plant can easily be removed from the Rootrainers and the soil or substrate can gently be washed away to score the whole root system. Rootrainers are kept in a rigid tray next to each other and can be kept on racks reducing space required. Cone-tainers™ (available in different sizes) are single cell plastic tubes that can be arranged in a tray allowing many plants in a limited space (Fig. 4.4). The tubes can easily be removed from the tray and submerged in water to rinse away the soil or medium and retrieve an intact plant for evaluation. Per tube, the nematodes from the soil can also individually be extracted to determine the final population. For Rootrainers and Cone-tainers, roots growing through the drainage holes are air pruned.

Fig. 4.1. **(A)** Closed containers with potatoes on white river sand for screening of potato cultivars for resistance against *Meloidogyne chitwoodi*. **(B)** Detail of closed container with egg masses of *Meloidogyne chitwoodi* (arrows) visible through the transparent container.

Fig. 4.2. The seedling growth pouch root-knot nematode screening system in a controlled environment chamber. **(A)** Young cowpea plants in seedling growth pouches arranged in hanging file folders following inoculation with *Meloidogyne incognita* second-stage juveniles. **(B)** Cowpea root system growing on the paper insert from a seedling growth pouch. **(C)** *M. incognita* egg mass on a root surface differentially stained blue with Brilliant Blue FCF (Erioglaucine) 35 days post inoculation. (Photographs courtesy of Philip A. Roberts, University of California Riverside, USA.)

The substrate or soil used in screening assays should allow normal root development and plant growth, nematode movement and host location, and should be easily removed from the roots to allow evaluation of the root system without damaging the plant and the roots. The latter is important as individual plants showing resistance can be further used for breeding purposes. When soil is used, high sand contents are preferred. Heat pasteurized or sterilized soil can be mixed with pure sand to increase the sand content. To allow comparison of data between years a standardized soil or substrate is recommended. A substrate used by Seinhorst *et al*. (1995) comprises 60% silver sand, 30% crushed ceramic material (hydro grains) and 10% clay powder (kaolin) supplemented with 1 g NPK (12:10:18) fertilizer kg^{-1}. This substrate proved to be successful for cyst and root-knot nematodes.

4.4.2 Inoculation

The optimum inoculum density depends on different aspects such as nematode species, type of inoculum (eggs, juveniles, cysts), pot size, plant species and environmental conditions. High inoculation densities may cause damage that adversely affects the development of plants and nematodes. For *H. avenae*, the formation of at least 20 white females per root system is considered the minimum for reliably distinguishing resistant and susceptible phenotypes and, thus, sufficient J2 to provide this number need to be inoculated (Andersen and Andersen, 1982). During inoculation, substantial loss of inoculum has been reported (Plowright and Gill, 1994; Mercer and Grant, 1995) and hatching of J2 may be limited. Preliminary tests to determine the optimum inoculation density under the same conditions as the screening test are recommended.

Inoculation is mostly done after germination and root establishment. The appearance of true leaves (two, four or more) can be used as standardized time for nematode inoculation and overcomes differences in seed germination. Soil can be inoculated before sowing or transplanting seedlings. Mixing and manipulating the infested soil may affect the nematodes, especially J2, adversely. When cysts are used as inoculum, inoculation before planting is recommended for cysts that respond to plant produced hatching factors (see Perry, Chapter 9,

Fig. 4.3. The four cell Rootrainer 'book' used to assess resistance of potato genotypes to potato cyst nematodes. **(A)** The Rootrainer (Tildenet Ltd, Bristol, UK) into which soil, cysts and tubers are planted. **(B)** An opened Rootrainer showing the root systems of potato plants. **(C)** Glasshouse assay using Rootrainers. (Photographs courtesy of Vivian Blok, James Hutton Institute, UK.)

Fig. 4.4. Screening assay for *Pratylenchus penetrans* and *Meloidogyne chitwoodi* resistance in cultivars of fodder radish (*Raphanus sativus*) using Cone-tainers™.

this volume). Cysts can be kept in a mesh buried in the soil to retrieve the inoculated cysts and differentiate them from newly formed cysts.

Inoculation on aerial plant parts with *Ditylenchus* spp. or *Aphelenchoides* spp. requires a humid environment when inoculation is done with a nematode suspension directly on the plants. A gelling agent (agar, carboxymethyl cellulose) may facilitate inoculation. High relative humidity should be maintained at least 1–2 days after inoculation (Plowright *et al*., 2002). For other nematode species, excessive water should be avoided for the first few days after inoculation.

In Petri dishes, inoculation can be done at root tips (Rivoal *et al*., 1991). In growth pouches, the inoculum can be distributed over the root system after which the pouches are kept in a horizontal position for 24 h (Atamian *et al*., 2012). In soil, inoculation can be done as soil drench but ideally the inoculum is distributed with a (micro)pipette in holes made around the stem base of the plants or injected in the soil profile with a side-bore syringe needle. The use of a syringe needle allows a more uniform inoculation in the soil profile. Therefore, the needle is inserted in the soil until 1 or 2 cm above the bottom of the pot/tube and gently retracted from the soil while releasing the nematode suspension. It is essential to check the needle regularly to avoid clogging. After inoculation, the holes are closed with soil.

The suspension of inoculum needs to be homogenized during inoculation. It is preferable to stir the suspension continuously using an air (aquarium) pump or a magnetic stirrer. Samples of the inoculum need to be checked at regular time intervals during the inoculation to ensure homogeneity.

4.4.3 Harvest and data acquisition

For breeding purposes plants need to be saved and replanted after assessment of the nematode infection. Therefore, procedures that allow careful handling and manipulation of the plants are preferred. Soilless methods (Petri dishes, growth pouches) facilitate easy observation and counting of females and cysts, and root-knot galling or egg masses of *Meloidogyne* spp. Plants kept in a sandy soil or substrate should be removed carefully from the pots after which the soil is gently washed away from the roots. Cysts and egg masses can detach from the roots during handling. Therefore, soil should be checked for the presence of newly formed cysts. Ideally, washing of the root system is done in a tray with water from which cysts can be retrieved, or over a sieve.

Galling induced by *Meloidogyne* spp. is not reliable to assess multiplication and reflects a plant response. Galling depends on nematode species, host plant species or cultivar and environmental conditions (Fassuliotis, 1979). Genes that mediate root galling but do not affect reproduction of *Meloidogyne* spp. have been identified (Garcia *et al*., 1996; Roberts *et al*., 2008). Galling might be correlated with reproduction, but this should be assessed separately before it can be used as scoring criteria. Counting egg masses of root-knot nematodes is useful when small or few galls are produced and when there is no correlation between galling and reproduction. For initial screening of large numbers of genotypes, counting of egg masses might be too time consuming but it should be done in later stages of the breeding programme when more accuracy is required (Starr and Mercer, 2009). Staining of egg masses with stains such as Erioglaucine (0.075 g l^{-1}) (Omwega, *et al*., 1988), Phloxine B (0.15 g l^{-1}) (Hussey and Boerma, 1981) and McCormick Shilling red food colour (20% v/v) (Thies *et al*., 2002) can facilitate counting. The average number of eggs per egg mass or eggs per gram root can further be used to rank plants, cultivars or breeding lines.

For migratory endoparasitic nematodes, assessment of reproduction requires (partial) destruction of the plants. Pudasaini *et al*. (2006) showed that for several crops more than 50% of the population of *P. penetrans* was present in the roots. Examining and extraction of nematodes from the whole root system is more accurate but not always feasible. Extraction of nematodes from roots (see Viaene *et al*., Chapter 2, this volume) might underestimate the final population due to extraction errors. Staining of nematodes (see Hallmann *et al*., Chapter 3, this volume) followed by counting is another option to evaluate reproduction.

Proper homogenization is a prerequisite when subsamples from roots, aerial plant parts or soil are taken. The fresh root or shoot weight and the total soil volume should be determined and the number of nematodes can be calculated per plant and per weight unit.

Multiplication on roots and tubers can be different and a combination of susceptible roots but resistant tubers has been reported (Brown *et al*., 2009). Yam roots rendered more *Scutellonema bradys* per g than tubers (Kwoseh *et al*., 2002).

Table 4.1. Standard scoring notation for relative susceptibility (OEPP/EPPO, 2006).

Relative susceptibility (%)	Score[a]
< 1	9
1.1–3	8
3.1–5	7
5.1–10	6
10.1–15	5
15.1–25	4
25.1–50	3
50.1–100	2
> 100	1

[a]Score of 9 indicates the maximum level of resistance.

The reproduction factor (*Rf*) obtained by dividing the final population (*Pf*) by the initial population (*Pi*) or reproduction index ($Ri = 100 \times Pf/Pi$) are used for assessment of resistance. *Rf* is density dependent and deteriorating roots and tubers can influence the results. For most assessments, a comparison with the susceptible control is made (relative susceptibility, *RS*): *Pf* test variety/*Pf* standard susceptible control variety ×100. To rank cultivars or breeding lines, a standard scoring notation based on *RS* can be used (Table 4.1) (OEPP/EPPO, 2006). For cyst nematodes the female index (females on test line/females on susceptible control ×100) can be determined and compared. Cyst content and viability can also be assessed but this is time consuming.

Results should be handled carefully if the coefficient of variation is too large. The coefficient of variation on the susceptible control should not exceed 35% (OEPP/EPPO, 2006).

4.5 Marker-assisted Selection

Marker-assisted selection (MAS), also known as marker-assisted breeding, is a selection process for genes that control traits of interest; for example, for resistance to pathogens it involves identifying a marker allele that is linked to disease resistance rather than the level of resistance. Screening based on genotype rather than phenotype is possible when resistance loci have been mapped in relation to DNA markers. Genotype is unaffected by environmental conditions and a single sample per breeding line can be used when there is no or limited genetic variability within the breeding line. MAS requires no inoculum, is non-destructive, rapid, more reliable and allows young plant tissue to be screened for resistance. It is very useful for rapid and efficient introgression of resistance genes found in wild or non-cultivated plant species and for pyramiding of genes. Use of MAS can prevent discarding large numbers of resistant breeding lines. MAS has been developed for important crop–nematode combinations and marker-assisted selection protocols are becoming more available. MAS can be employed at any stage of a plant breeding programme but has great advantage in early screening by eliminating undesirable gene combinations (Ragimekula *et al*., 2012). MAS selected genotypes require phenotypic verification to ensure there is no loss of association between the marker and the resistance gene (Blok *et al*., 2018).

4.6 References

Andersen, K. and Andersen, S. (1982) Classification of plants resistant to *Heterodera avenae*. *EPPO Bulletin* 12, 435–437. DOI: 10.1111/j.1365-2338.1982.tb01826.x

Atamian, H.S., Roberts, P.A. and Kaloshian, I. (2012) High and low throughput screens with root-knot nematodes *Meloidogyne* spp. *Journal of Visualized Experiments* 61, e3629. DOI: 10.3791/3629

Aubert, V. (1986) Hatching of the carrot cyst nematodes. In: Lamberti, F. and Taylor, C.E. (eds) *Cyst Nematodes*. Plenum Press, New York, New York, USA, pp. 347–348.

Balasubramanian, P. and Ramakrishnan, C. (1983) Resistance to the reniform nematode *Rotylenchulus reniformis* in tomato. *Nematologia Mediterranea* 11, 203–204.

Bird, A.F. and Wallace, H.R. (1965) The influence of temperature on *Meloidogyne hapla* and *M. javanica*. *Nematologica* 11, 581–589. DOI: 10.1163/187529265X00726

Blok, V.C., Tylka, G.L., Smiley, R.W., de Jong, W.S. and Daub, M. (2018) Resistance breeding. In: Perry, R.N., Moens, M. and Jones, J.T. (eds) *Cyst Nematodes*. CAB International, Wallingford, UK, pp. 174–214.
Boerma, H.R. and Hussey, R.S. (1992) Breeding plants for resistance to nematodes. *Journal of Nematology* 24, 242–252.
Brown, C.R., Mojtahedi, H., Zhang, H.L. and Riga, E. (2009) Independent resistant reactions expressed in root and tuber of potato breeding lines with introgressed resistance to *Meloidogyne chitwoodi*. *Phytopathology* 99, 1085–1089. DOI: 10.1094/PHYTO-99-9-1085
De Waele, D. and Elsen, A. (2002) Migratory endoparasites: *Pratylenchus* and *Radopholus* species. In: Starr, J.L., Cook, R. and Bridge, J. (eds) *Plant Resistance to Parasitic Nematodes*. CAB International, Wallingford, UK, pp. 175–206.
Fassuliotis, G. (1979) Plant breeding for root-knot nematode resistance. In: Lamberti, F. and Taylor, C.E. (eds) *Root-knot Nematodes* (Meloidogyne *species*) *Systematics, Biology and Control*. Academic Press, London, UK, pp. 425–453.
Faulkner, L.R., Bower, D.B., Evans, D.W. and Elgin, J. (1974) Mass culturing of *Ditylenchus dipsaci* to yield large quantities of inoculum. *Journal of Nematology* 6, 126–129.
Garcia, G.M., Stalker, H.T., Shroeder, E. and Kochert, G. (1996) Identification of RAPD, SCAR, and RFLP markers tightly linked to nematode resistance genes introgressed from *Arachis cardenasii* into *Arachis hypogaea*. *Genome* 39, 836–845. DOI: 10.1139/g96-106
Greco, N. (1981) Hatching of *Heterodera carotae* and *H. avenae*. *Nematologica* 27, 366–371. DOI: 10.1163/187529281X00377
Greco, N., Vito, M.D. and Lamberti, F. (1986) Studies on the biology of *Heterodera goettingiana* in southern Italy. *Nematologia Mediterranea* 14, 23–29.
Hussey, R.S. and Barker, K.R. (1973) A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Disease Reporter* 57, 1025–1028.
Hussey, R.S. and Boerma, H.R. (1981) A greenhouse screening procedure for root-knot nematode resistance in soybean. *Crop Science* 21, 794–796.
Hussey, R.S. and Janssen, G.J.W. (2002) Root-knot nematodes: *Meloidogyne* species. In: Starr, J.L., Cook, R. and Bridge, J. (eds) *Plant Resistance to Parasitic Nematodes*. CAB International, Wallingford, UK, pp. 43–70.
Ishibashi, N., Kondo, E., Muraoka, M. and Yokoo, T. (1973) Ecological significance of dormancy in plant-parasitic nematodes. I. Ecological difference between eggs in gelatinous matrix and cysts of *Heterodera glycines* Ichinohe (Tylenchida: Heteroderidae). *Applied Entomology and Zoology* 8, 53–63.
Janssen, R., Bakker, J. and Gommers, F.J. (1987) Circumventing the diapauses of potato cyst nematodes. *Netherlands Journal of Plant Pathology* 83, 107–113. DOI: 10.1007/BF02000561
Khan, A., Wesemael, W.M.L. and Moens, M. (2014) Influence of temperature on the development of the temperate root-knot nematodes *Meloidogyne chitwoodi* and *M. fallax*. *Russian Journal of Nematology* 22, 1–9.
Kwoseh, C., Plowright, R.A. and Bridge, J. (2002) The yam nematode: *Scutellonema bradys*. In: Starr, J.L., Cook, R. and Bridge, J. (eds) *Plant Resistance to Parasitic Nematodes*. CAB International, Wallingford, UK, pp. 221–228.
Masler, E.P. and Perry, R.N. (2018) Hatch, survival and sensory perception. In: Perry, R.N., Moens, M. and Jones, J.T. (eds) *Cyst Nematodes*. CAB International, Wallingford, UK, pp. 44–73.
Mercer, C.F. and Grant, J.L. (1995) Resistance of the white clover variety G49 and its parent lines to stem nematode (*Ditylenchus dipsaci*). *New Zealand Journal of Agricultural Research* 38, 495–499.
Mizukubo, T. and Adachi, H. (1997) Effect of temperature on *Pratylenchus penetrans* development. *Journal of Nematology* 29, 306–314.
OEPP/EPPO (2006) Testing of potato varieties to assess resistance to *Globodera rostochiensis* and *Globodera pallida*. *OEPP/EPPO Bulletin* 36, 419–420. DOI: 10.1111/j.1365-2338.2006.01032.x
Omwega, C.O., Thomason, I.J. and Roberts, P.A. (1988) A non-destructive technique for screening bean germ plasm for resistance to *Meloidogyne incognita*. *Plant Disease* 72, 970–972.
Peng, Y.L. and Moens, M. (1999) Effects of surface sterilisation and cold storage on *in vitro* behaviour of *Pratylenchus penetrans*. *Nematology* 1, 647–653. DOI: 10.1163/156854199508603
Phillips, M.S., Forrest, J.M.S. and Wilson, L.A. (1980) Screening for resistance to potato cyst nematode using closed containers. *Annals of Applied Biology* 96, 317–322. DOI: 10.1111/j.1744-7348.1980.tb04782.x
Plowright, R.A. and Gill, J.R. (1994) Aspects of resistance in deepwater rice to the stem nematode *Ditylenchus angustus*. *Fundamental and Applied Nematology* 17, 357–367.
Plowright, R.A., Caubel, G. and Mizen, K.A. (2002) *Ditylenchus* species. In: Starr, J.L., Cook, R. and Bridge, J. (eds) *Plant Resistance to Parasitic Nematodes*. CAB International, Wallingford, UK, pp. 107–139.
Pudasaini, M.P., Schomaker, C.H., Been, T.H. and Moens, M. (2006) Vertical distribution of the plant-parasitic nematode, *Pratylenchus penetrans*, under four field crops. *Phytopathology* 96, 226–233. DOI: 10.1094/PHYTO-96-0226

Ragimekula, N., Varadarajula, N.N., Mallapuram, S.P., Gangimeni, G., Reddy, R.K. and Kondreddy, H.R. (2012) Marker assisted selection in disease resistance breeding. *Journal of Plant Breeding and Genetics* 1, 90–109.
Rivoal, R., Lasserre, F., Hullé, M. and Doussinault, G. (1991) Evaluation de la résistance et de la tolérance à *Heterodera avenae* chez le blé par tests miniaturisés. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent* 56, 1281–1292.
Roberts, P.A. (2002) Concepts and consequences of resistance. In: Starr, J.L., Cook, R. and Bridge, J. (eds) *Plant Resistance to Parasitic Nematodes*. CAB International, Wallingford, UK, pp. 23–41.
Roberts, P.A., Matthews, W.C., Ehlers, J.D. and Helms, D. (2008) Genetic determinants of differential resistance to root-knot nematode reproduction and galling in lima bean. *Crop Science* 48, 553–561. DOI: 10.2135/cropsci2007.07.0384
Seinhorst, J.W., Oostrom, A., Been, T.H. and Schomaker, C.H. (1995) Relative susceptibilities of eleven potato cultivars and breeders' clones to *Globodera pallida* pathotype Pa3, with a discussion of the interpretation of data from pot experiments. *European Journal of Plant Pathology* 101, 457–465. DOI: 10.1007/BF01874859
Sijmons, P.C., Grundler, F.M.W., von Mende, N., Burrows, P.R. and Wyss, U. (1991) *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *The Plant Journal* 1, 245–254. DOI: 10.1111/j.1365-313X.1991.00245.x
Speijer, P.R. and De Waele, D. (1997) *Screening of Musa Germplasm for Resistance and Tolerance to Nematodes.* International Plant Genetic Resources Institute, Montpellier, France.
Starr, J.L. and Mercer, C.F. (2009) Development of resistant varieties. In: Perry, R.N., Moens, M. and Starr, J.L. (eds) *Root-knot Nematodes*, CAB International, Wallingford, UK, pp. 326–337.
Starr, J.L., Cook, R. and Bridge, J. (eds) (2002) *Plant Resistance to Parasitic Nematodes*. CAB International, Wallingford, UK.
Thies, J.A., Merrill, S.B. and Corley, E.L. (2002) Red food coloring stain: new, safer procedures for staining nematodes in roots and egg masses on root surfaces. *Journal of Nematology* 34, 179–181.
Trudgill, D.L. (1991) Resistance to and tolerance of plant parasitic nematodes in plants. *Annual Review of Phytopathology* 29, 167–192. DOI: 10.1146/annurev.py.29.090191.001123
Wang, H., Mao, J., Li, R., Luo, D., Zhao, G. and Li, H. (2016) A culture technique for *Ditylenchus destructor* on sweet potato (*Ipomoea batatas*). *Nematology* 18, 501–503. DOI: 10.1163/15685411-00002969
Wesemael, W.M.L., Taning, L.M., Viaene, N. and Moens, M. (2014) Life cycle and damage of the root-knot nematode *Meloidogyne minor* on potato, *Solanum tuberosum*. *Nematology* 16, 185–192. DOI: 10.1163/15685411-00002756
Xu, L., Li, S., Shabala, S., Jian, T. and Zhang, W. (2019) Plants grown in Parafilm-wrapped Petri dishes are stressed and possess altered gene expression profile. *Frontiers in Plant Science* 10, 1–12. DOI: 10.3389/fpls.2019.00637
Yokawa, K., Kagenishi, T., Kawano, T., Mancuso, S. and Baluška, F. (2011) Illumination of *Arabidopsis* roots induces immediate burst of ROS production. *Plant Signaling & Behavior* 6, 1460–1464. DOI: 10.4161/psb.6.10.18165
Zhen, F., Agudelo, P. and Gerard, P. (2012) A protocol for assessing resistance to *Aphelenchoides fragariae* in *Hosta* cultivars. *Plant Disease* 96, 1438–1444. DOI: 10.1094/PDIS-10-11-0895-RE

5 Handling, Fixing, Staining and Mounting Nematodes

JONATHAN D. EISENBACK[1] AND DAVID J. HUNT[2]

[1]*Virginia Tech, Blacksburg, Virginia, USA;* [2]*CABI, Egham, Surrey, UK*

5.1 Introduction

Numerous techniques for handling, fixing, staining and mounting nematodes have been published during the development of the science of nematology over the last 60 years (Thorne, 1961; Goodey, 1963; Zuckerman *et al*., 1985; Hooper, 1986; Southey, 1986; Sulston and Hodgkin, 1988; De Ley *et al*., 1995; Kleynhans, 1999; Pest CABweb, 2002; Ravichandra, 2002; Coyne *et al*., 2007; van Bezooijen, 2006). This chapter includes those techniques that have been shown to be widely adapted and least hazardous to the user and the environment. Additional techniques that are useful or better for particular nematodes can easily be adopted from other sources; however, these basic procedures are a good starting point for new students of nematology.

5.2 Handling Nematodes

Because nematodes are usually microscopic, special tools and techniques are necessary for manipulating them for various procedures. Handling nematodes is most commonly necessary for picking individuals out of an extraction in order to make a mount for microscopic observation including identification. Special tools are necessary for picking individual specimens and arranging them on the slide, for selecting individuals for additional procedures, such as preparation for scanning (SEM) or transmission (TEM) electron microscopy or other special applications, and for establishing cultures by picking out individuals to make a pure culture either in a Petri dish or to establish a pot culture.

5.2.1 Nematode picks

Picking nematodes is a tiring and time-consuming task; therefore, selection of a good pick is very important for handling individual nematodes efficiently. The pick must have a small diameter and be slightly curved and rigid. Picks are generally not available for purchase but have to be hand-crafted. They can be made by attaching a small hair, cactus spine, nylon fibre or bamboo sliver to a wooden handle with waterproof glue, or by using a small diameter insect-mounting pin (e.g. a stainless steel micropin) or acupuncture needle that is attached to the handle of a hobby knife. Sometimes the hair of choice may be very specific, i.e. the eyelash of a female hog, a cat's whisker, or the hair from a man's beard, but often hairs are too flexible, and spines

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
10.1079/9781786391759.0005

and slivers of bamboo are too straight, although the latter can be shaved using a scalpel to form a curved and very slender tip. Likewise, cat whiskers are spatulate and therefore useful for scooping up nematode eggs, but not for picking up nematodes. In the case of the micropin, the blunt end may be carefully inserted into the square end of a matchstick (entomological forceps are very useful here because of their curved and broad ends, but watchmaker's forceps will suffice) or glued to the end of a suitable handle, such as a toothpick. Insect micropins are available in different diameters and their point may, using fine forceps, be bent or hooked to a suitable angle depending on intended use. They are also rigid, enabling them to pull large nematodes through the meniscus without them being dragged off by surface tension. A good nematode pick can also be made from a dental root canal file attached to a handle that is specially made to hold it (Eisenback, 2015). Mounted micropins and dental root canal files make for excellent tools that enable picking, perineal pattern or cyst cone cleaning, dissection to study genital organ structure or spicule form, etc., and cutting, thereby facilitating *en face* mounts or transverse sections to study lateral field structure, etc. Figure 5.1 shows an endodontic file (H- type #25 – 21 mm long) fitted into a special endohandle (Brunel Microscope Secure, n.d.).

The endohandle provides a sure grip and its pen-like shape fits in the hand comfortably and gives good control for picking even the smallest nematodes. In addition, it can be used for hours without tiring your hand. The anterior end is fitted with a locking chamber that holds the endodontic file securely. These files are inexpensive, unbreakable, autoclavable and give fine tactile control. However, being files their rough edges

Fig. 5.1. Package of five universal endohandles that are useful holders for the endodonic H-type files (left) and a single handle with an attached H-type #25 file (21 mm long) that has been sanded smooth with a sharpening stone and slightly curved to make a nematode handling pick.

must be smoothed with a honing stone, fine sandpaper or emery cloth. After the files are smooth and finely pointed, forceps can be used to bend the point at an angle or into a smooth arc. Several files can be modified for use in carrying out different tasks, such as picking nematodes, cleaning the inside of perineal patterns, mounting cyst vulval cones in glycerin jelly, extracting stylets and spicules, and mounting specimens for observation in the scanning electron microscope. Such tools may not be readily available locally, however, requiring the user to modify and adapt the principle using other materials.

5.2.2 Nematode picking technique

With the aid of a low magnification stereoscopic dissecting microscope, a single nematode in a liquid suspension is selected and lifted off the bottom of the dish with a gentle, nearby swish of the pick. While in suspension, the pick is placed under the middle region of the nematode, which is pushed up toward the surface where it is carried through the surface tension of the water with one smooth, but rapid, flick. If the liquid is deep or the focal area is shallow, one hand is used to keep the specimen in focus by turning the focusing knob, while the other hand with the nematode pick lifts the specimen and transfers it to another drop of liquid of choice. With practice, it is possible to pick up two or more nematodes on each trip. When picking nematodes after processing to glycerin, the more rigid picks perform better, because hair-based picks are too flexible for the viscous medium.

5.2.3 Pipettes

Pipettes are easier and more useful for transferring numerous individuals from one solution to another. Glass is preferable to plastic because the nematodes may stick to the latter. A standard glass Pasteur pipette can be fashioned into a micropipette by melting the narrow end over a flame and rapidly pulling it to form a narrower diameter. In order to select a mass group of nematodes, they can be suspended and moved to the centre by swirling them in small, concentric circles in a small dish such as a Syracuse watch glass or Petri dish.

5.2.4 Forceps

Forceps with fine points, such as watchmaker's forceps, are useful to tease galled root tissue apart to remove root-knot females and other swollen females, i.e. cysts, from roots. One hand holds the tissue in place with a dissecting needle while the other hand macerates the tissue with the forceps. Once freed from the plant root, the female can be carefully plucked from the water with forceps. To avoid squeezing the forceps too tightly and breaking the female, the tip of the index finger can be placed in between the two sides of the forceps which are then gently squeezed together with the thumb and middle finger.

5.2.5 Aspirator

Cyst nematodes, root-knot females, and other nematodes can be selected from water with a small aspirator that is controlled by a rubber tube held in between the teeth and sealed with the lips (Fig. 5.2). A small glass pipette attached to a rubber tube is used to suck specimens into the bottle. One end of the pipette can be gently heated over a flame and stretched to a diameter that is appropriate for the specimens that are being selected.

5.2.6 Micro-chambers

Various micro-chambers are useful for handling nematodes. A chamber that is useful for processing nematodes for SEM, TEM, or making permanent mounts can be made out of a BEEM® capsule by removing its conical end with a razor blade (Eisenback, 1985). Perforate its cap with a hole punch and then place it onto the bottom of the capsule with a small square of very fine cloth sandwiched in between the lid and the capsule (Fig. 5.3). A second lid can be fashioned in the same manner for the top of the chamber in order to contain the specimens if the chamber turns over while it is being used.

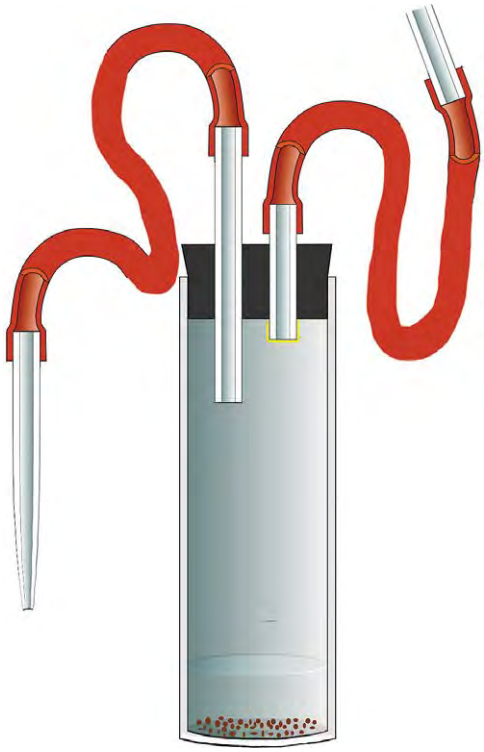

Fig. 5.2. Drawing of an aspirator used to pick large nematode such as cysts and adult root-knot females. It can be made by inserting two short glass tubes through a rubber stopper plugging a small glass vial. A short length of rubber tubing is attached to each glass tube, one with a glass pipette that has been heated over a flamed and pulled to an appropriate diameter, and a second tube fitted with a mouthpiece used to produce a vacuum in the glass vial.

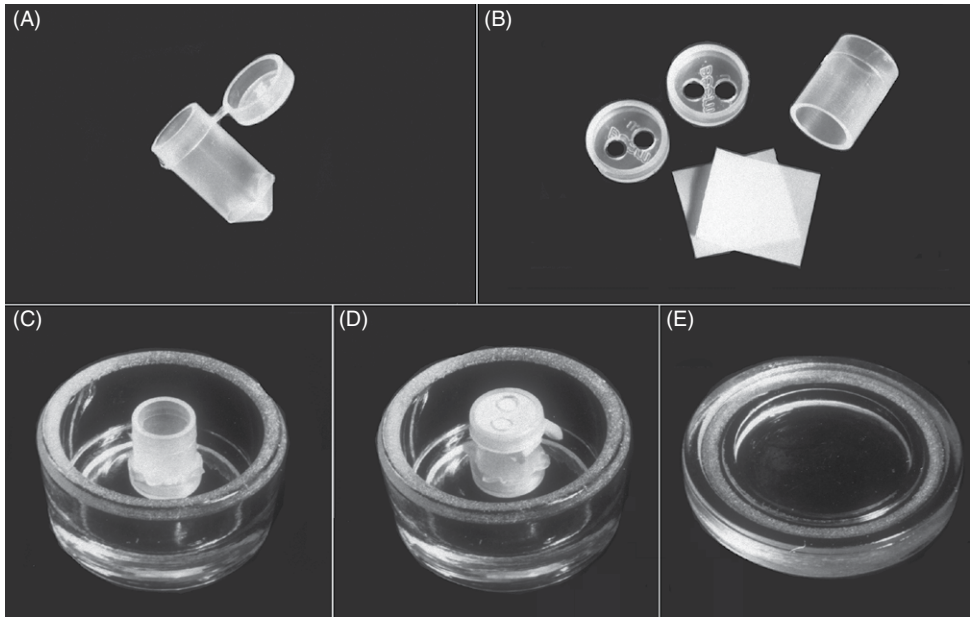

Fig. 5.3. **(A)** A BEEM capsule**. (B)** Two lids with holes punched in them, the cone bottom removed with a razor blade, and two small square pieces of fine mesh cloth that makeup the container. **(C)** A capsule in a Stendor dish without the cap on the capsule or the dish. **(D)** The capsule with the cap in place in a Stendor dish. **(E)** The lid of the Stendor dish. (From Eisenback, 1985.)

5.2.7 Useful dishes

Small dishes are very helpful for handling nematodes. The standard Bureau of Plant Industry (BPI) dish and the larger Syracuse watch glass are very useful (Fig. 5.4).

5.3 Killing and Fixing Techniques

Adequate fixing techniques for nematodes are necessary because they start to deteriorate rapidly as soon as they are killed. The fixation process should preserve the organs, the form and dimensions of the nematode in as life-like condition as possible in order to avoid complications and potential error with subsequent identification. In order to observe and accurately measure nematodes, they must be fixed using proven materials and techniques, preferably following a standard method. Nematodes are best killed by using relatively gentle heat (i.e. 55–60°C) as this does not risk disrupting the body contents. Specimens killed by this means assume an unnatural, but often characteristic 'relaxed' shape, which can help in their identification. Nematodes can either be killed first and then fixed or killed and fixed at the same time. The method chosen is usually down to personal preference, although if hot fixative is used, appropriate health and safety procedures must be followed as fixatives are usually toxic and/or irritant in nature. High-quality preservation is important so that accurate measurements and observations can be made for precise identification or description of the nematodes.

Treating complete nematode suspensions is generally the most convenient method of killing and fixing. After extraction, the nematode suspension is concentrated into a small volume of water (< 20 ml) by settling and then decanting, or siphoning, or centrifuging. The reduced volume can then be heated to a temperature of 55–60°C directly over a flame, a hot plate or in an oven; or by partially immersing a container with the nematode suspension in a large volume of water at approximately 80–90°C (off the boil) for a few minutes (the temperature of the extract can be checked with a thermometer if required). Whichever technique is used, the nematodes must *not* be boiled or over heated. After killing in this way, the sample is left to cool and then the whole nematode suspension is fixed by either adding an equal volume of 'double strength' fixative or an excess of 'normal strength' fixative. Alternatively, individual nematode specimens can be picked out of the suspension and transferred into cold fixative at the 'normal strength' shown below. Single, or small numbers of nematodes, can be killed and fixed in a drop of water on a glass slide in the same way as above, but overheating (and boiling) is a real danger and should be avoided at all costs.

There are several fixatives in common use, most involving formaldehyde (*care*!) either on its own or in a blend with various additives. Fixing and killing of nematodes in suspension can be done using formaldehyde or formaldehyde acetic (FA) 4:1 (Seinhorst, 1966). Double strength fixative is heated to 80–90°C (just off the

Fig. 5.4. A Syracuse watch glass; a Bureau of Plant Industry (BPI) dish is similar in shape but only 2.5 cm diam.

boil) in a fume cupboard and then poured into an equal or slightly smaller volume of nematode suspension. Some popular fixatives are listed in Table 5.1.

No single fixative is perfect. For example, 2–4% formaldehyde tends to coagulate proteins and form a dark, blocky appearance that obscures body contents until the specimens have been cleared by processing to glycerin; triethanolamine formaldehyde (TAF) provides and maintains a very life-like appearance, although some sclerotized structures can become very transparent over time and some degradation of the external cuticle may occur. On the other hand, TAF will not evaporate entirely, the nematodes remaining in the triethanolamine residue, although a similar 'insurance policy' can be achieved by adding a trace of glycerin to the other fixatives. Nematodes in fixative should be left for 12 h or overnight before processing further to allow adequate penetration of the tissues. Nematodes may also be fixed in DESS (dimethyl sulphoxide, disodium EDTA, and saturated NaCl) (Yoder *et al*., 2006). This fixative also facilitates subsequent molecular studies of the preserved material, something made more difficult if formaldehyde is used in the fixative. There are several protocols for making DESS, the simplest being to make a large volume as given in Table 5.2. The quantity of NaCl should be sufficient to form a saturated solution. See also: www.faculty.ucr.edu/~pdeley/lab/melissa/DESS_protocol_f.doc.

5.3.1 Permanent mounts

Permanent mounts usually involve killing the nematodes with a hot fixative and gradually replacing the water with anhydrous glycerin. Several variations of preparing nematodes for permanent mounts have been published (Baker, 1953; Seinhorst, 1959; Thorne, 1961; De Grisse, 1969; Esser, 1973; Ryss, 2003) but most are similar to that proposed by Seinhorst (1962). Various techniques of fixation may affect measurements and other morphological features when compared to unfixed specimens (Lamberti and Sher, 1969; Stone, 1971; Esser, 1974; Fagerholm, 1979; Curran and Hominick, 1981; Stynes and Bird, 1981; Boag, 1982; Grewal *et al*., 1990).

Table 5.1. Popular fixatives.

Fixative	Reagents	Quantity[a]
Formaldehyde	40% formaldehyde [= formalin]	2% [5%]
FA 4:1	40% formaldehyde	10 ml
	Glacial acetic acid	1 ml
	Distilled water	89 ml
FP 4:1	40% formaldehyde	10 ml
	Propionic acid	1 ml
	Distilled water	89 ml
TAF	40% formaldehyde	7 ml
	Triethanolamine	2 ml
	Distilled water	91 ml

[a]Single strength. Double strength solutions can made by halving the amount of water.
FA, formaldehyde acetic; FP, formaldehyde propionic; TAF, triethanolamine formaldehyde.

Table 5.2. Protocol for making up DESS.

Reagents	Volume
Dimethyl sulphoxide (DMSO)	400 ml
0.5 M Disodium EDTA	1000 ml
NaCl solution	*ca* 300 g
Deionized water	600 ml

5.3.1.1 Seinhorst slow method

Seinhorst (1962) gave details of a slow method for making permanent mounts in pure glycerin.

- Pick nematodes into 10 drops of water.
- Add 10 drops of hot (80°C) FA 4:1 fixative to relax the specimens.
- Fix for 48 h.
- Rinse specimens in distilled water.
- Place in Seinhorst I solution (20 parts 95% ethanol; 1 part glycerin; 79 parts water) in a BPI watchglass (excavated glass block or solid watchglass).
- Place open BPI watchglass in a desiccator surrounded by 95% ethanol.
- Incubate at 40°C for 12 h.
- Add 20 drops of Seinhorst II solution (95 parts 95% ethanol; 5 parts glycerin).

5.3.1.2 Ryss express method

A quick method (Ryss, 2003) for preparing permanent mounts takes advantage of Eppendorf tubes and programmable thermal controllers that are commonplace in laboratories that utilize PCR for molecular techniques of studying nematodes.

- Pick nematodes into a small drop of water in an Eppendorf tube (0.5 ml).
- Fill another Eppendorf tube (0.5 ml) with 4% formalin.
- Place tube with formalin into a beaker with hot water (95°C).
- Transfer the hot formalin to the Eppendorf with nematodes.
- Close cap and shake to prevent the specimens from sticking to the tube.
- Place into a programmable thermal controller with the following schedule:
  - 95°C for 2 min;
  - 65°C for 10 min;
  - 75°C for 10 min;
  - 85°C for 10 min; and
  - 95°C for 10 min.
- Remove from thermocycler and allow to reach room temperature.
- Shake contents and pour into a BPI watch glass.
- Rinse the tube several times with distilled water and pour into the watch glass.
- Pick the nematodes from the watch glass into a glass cavity slide filled with a 1:20 mixture of distilled water and glycerin.
- Place the cavity slide on a hotplate at 70°C for 15–20 min, the surface will change from a wave to a smooth plane when the water is gone.
- If shrinkage of the specimens has occurred, increase the hotplate to 75–80°C and move the cavity slide to a room temperature surface and back to the hotplate three to five times until the shrinkage is gone.

5.3.1.3 Simple evaporation method

Nematodes may be placed in a 5% solution of glycerin in the chosen fixative, the solution being left in a small, partially covered container to allow evaporation to proceed, eventually leaving the nematodes in pure glycerin. The process may be accelerated or retarded by varying the size of the opening – nematodes with a weaker cuticle benefit from a longer and slower process, thus avoiding distortion and 'ribbon-like' nematodes. In more humid climates it may be necessary to finish the process in a laboratory oven/incubator set to a low, but suitable temperature. If the process occurs too rapidly, ribbon-like nematodes may result as the internal water is removed faster than the glycerin can penetrate. This also may occur with thick-cuticled nematodes

such as criconematids or hoplolaimids. Usually, this problem can be reversed by placing the nematodes in a drop of glycerin on a slide and very gently applying heat either from a spirit lamp flame or a slide warming plate, checking often under the stereomicroscope to assess how far inflation has progressed.

5.3.2 Fixing for SEM and TEM

Glutaraldehyde in various strengths and buffers is the most commonly used fixative for preparing nematodes for electron microscopy (SEM and TEM). Sequential fixation of specimens for SEM is based on the idea that as the fixation wave front passes through the tissue, it becomes more difficult to penetrate, and the interior of the specimen remains less fixed (Eisenback, 1985). Gradually increasing the fixative strength allows for the fixative to penetrate deeper into the tissues, and they gradually become harder and harder as the strength of the fixative increases. This type of fixation is always carried out in the cold (4–8°C) so that the nematodes will relax in a straightened shape and body excretions will be minimized, otherwise important features of the head may be obscured by secretions from the amphids.

5.4 Processing Nematodes for Temporary and Permanent Mounts

5.4.1 Temporary mounts

Temporary mounts of nematodes are used for observing nematodes that have been picked from a solution with a compound microscope for additional observations at higher magnification. They usually keep for a few hours but can be sealed and refrigerated to last 2–3 days.

5.4.1.1 Support rings

Rings of fingernail polish (or other paints/sealers) or wax rings can be used to support the cover slip and to prevent it from flattening or crushing the delicate nematode specimens. The rings of sealer or nail polish are made slightly smaller than the diameter of the nematode with a fine tipped artist's brush. If the ring is too high the specimen will float and cannot be viewed with an oil-immersion lens because it puts a slight pressure on the cover slip. Wax rings are more forgiving because they can be reduced in thickness by applying slight pressure on the cover slip while the wax is gently warmed over a heat source.

Temporary slides made with the rings involves picking the specimens into a small drop of tap water on a slide, gently relaxing them over a flame, and then transferring them into a drop of fixative on a ringed slide and adding a cover slip on top. The excess fixative is drawn off from the slide and the cover slip is sealed with a coating of nail polish or other paint or wax that is allowed to dry before observation. These slides last for 3–7 days or longer if stored in a refrigerator. The pitfalls of making temporary slides in this manner include the extra handling of the nematodes from water to fixative, the difficulty of making a ring that is the proper height, and placing the cover slip on correctly so that air bubbles are minimized and the specimens do not wash away from the ring.

5.4.1.2 Agar pads

The easiest and most efficient way to make a temporary slide is to use a pad of 5% water agar (Fig. 5.5) (Eisenback, 2012). The pad is made by placing three slides on the laboratory bench top with the two outer slides containing a layer of thick tape. Add a small drop of melted 5% water agar in the centre of the middle slide and place a fourth slide perpendicular to it on top of the agar, flattening it into an evenly thick pad. Nematode specimens are picked into a small drop of tap water on a cover slip. After they are pressed to the bottom of the drop, the cover slip is inverted and placed on the agar pad. Nearly every slide made with this technique is perfect. The specimens are lying on their lateral side and they are almost entirely in one plane. A small amount of sodium azide (0.1 M) can be added to the agar to paralyse the specimens that can be observed while still alive but not moving.

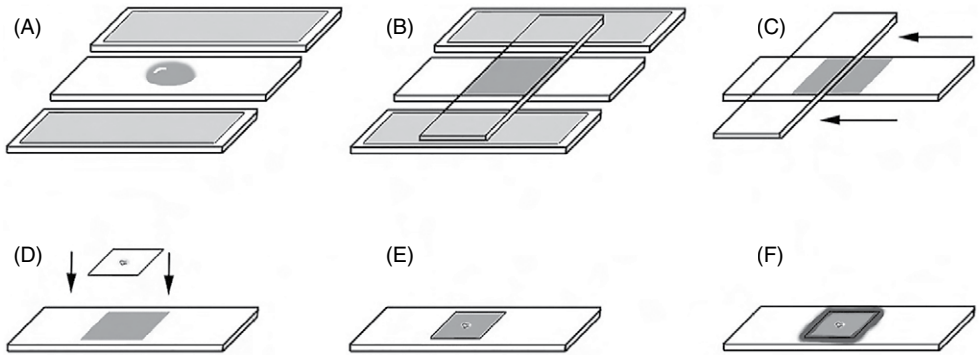

Fig. 5.5. Diagram showing how glass slides with an agar pad are made. **(A)** Three glass slides are arranged parallel to each other. The outer two slides have a piece of tape attached. The centre slide receives a hot drop of 4% water agar. **(B)** A fourth slide is placed at right angles to the bottom three slides and flattens the drop of agar. **(C)** After the agar solidifies, the two slides are gently slid apart and the agar pad remains attached to one slide. **(D)** Nematodes are picked by hand and placed in a small drop of tap water on a square cover slip that is inverted and placed straight down onto the agar pad **(E)**. The cover slip is sealed with a small layer of nail polish and allowed to dry. (After Eisenback, 2012.)

5.4.2 Slide making

Make permanent slides from either the Seinhorst slow method (see Section 5.3.1.1) or the Ryss express method (see Section 5.3.1.2) by placing a small drop of anhydrous glycerin on a glass slide and pick the nematodes into the drop. Ensure that the specimens are lying on the bottom of the drop and arranged neatly. Select one of the following three methods to support the cover slip.

5.4.2.1 Glass wool supports or Ballotini beads

With a dissecting microscope, three short lengths of glass fibres or Ballotini beads of varying diameters are compared to the nematode specimens that are slightly smaller in diameter and used to support the cover slip. The fibres or beads are immersed in glycerin and at least three arranged equidistantly near the edges of the drop of glycerin, the nematodes being transferred into the drop, arranged parallel with each other in the centre of the drop and covered with a cover slip. After the cover slip settles on top of the specimens, and makes contact with the glass fibres, the excess glycerin is wicked away with small triangular pieces of filter paper, and the slide is sealed with nail polish or other sealer.

5.4.2.2 Fingernail polish or paint rings

Since the special mixtures of ZUT and Glyceel are no longer commercially available, fingernail polish or nail varnish (formulas with acrylic hardeners are best and can be obtained in colourless or tinted varieties) or other suitable paint is a useful substitute for making ring supports for cover slips. A good brand for this purpose is *Sally Hansen Hard as Nails* 'Hardener' (Fig. 5.6) that is slightly tinted or not. It is best applied with a small artists paint brush '0' to '000' and a specially manufactured slide ringer (Brunel Microscope) (Fig. 5.7). The height of the ring works best if it is slightly lower than the diameter of the nematode because if it is too low the nematodes will be slightly flattened, and if it is too high the specimens will be floating and nearly impossible to image under high power because they will move out from under the objective, which places a slight pressure downward on the mount.

5.4.2.3 Wax support rings

Wax rings have been useful replacements for hard sealers like nail polish and make the process of preparing slides a little easier. They are made by heating either a small diameter, thin-walled copper tube, aluminium pipe, suitable

Fig. 5.6. A bottle of Sally Hansen 'Hard as Nails' nail polish used to seal the cover slip onto the slide.

Fig. 5.7. A commercially made apparatus for ringing slides.

diameter cork borer or Pyrex glass tube over a heat source and melting the surface of a wax mixture and transferring the wax to a glass slide. The ring is filled with glycerin and the specimens are transferred to the centre of the drop, arranged parallel with each other, and the cover slip is dropped into place. The slide is put onto a warming tray where the cover slip gradually sinks down until it comes into contact with the nematodes. The excess glycerin is absorbed with triangular pieces of filter paper, and the slide is sealed with wax. A fast technique that utilizes wax supports may be useful for making slides of numerous nematodes for collections (Ryss, 2017).

5.5 Mounting *en face* Views and Cross-sections

Mounting nematodes *en face* and making cross-sections along the body may be necessary to detect certain morphological characters such as cephalic framework, amphidial openings or lines in the lateral field. *En face*

views can be made either by mounting glycerin infiltrated specimens into a drop of glycerin jelly or by sandwiching wet, fixed specimens between slabs of water agar. Cross-sections can either be done by hand cutting specimens in glycerin jelly or by embedding in wax or plastic and cutting into thin sections with a microtome.

The head morphology of a nematode may be taxonomically important and observation is difficult unless the specimen is specially prepared for an *en face* view. Because these mounts are almost impossible to make with a microtome, instead they are cut by hand. This technique was first implemented by Cobb (1920), embellished by Chitwood and Wehr (1934), and further refined by Basir (1949). In this method, the nematode is first fixed and processed into glycerin by the Seinhorst (1962) or Ryss (2003) method and then transferred into a small streak of glycerin jelly on a glass slide, cut with a sharp eye-knife or scalpel, and arranged in an upright position with a wire nematode pick (Fig. 5.8). Additional cross-sections along the length of the entire body is possible with this technique. The position of the specimen on the slide is marked with a permanent marking pen on the underneath side (Basir, 1949) so that it can be more easily found and observed with a compound microscope.

Another useful technique to observe *en face* views of large nematodes without the necessity of cutting the specimen uses a 1.7% water agar block to support formalin- or glutaraldehyde-fixed nematodes in an upright position (Esser, 1986; Fig. 5.9). Cut a layer of 3 mm thick agar into a 12 × 12 mm square block. Lay the agar on a clean microscope slide and cut a 3 mm slice from one end. Lay the slice over on its side and place three to four nematodes on the top of the slice with their head near the cut edge. Stand the specimens perpendicular to the slide by returning the cut slice to its original position. Place a small drop of tap water on a cover slip, invert the cover slip, and place it on top of the agar block. View the specimens with an oil-immersion lens. If the nematodes are tilted or too deep in the agar, take off the cover slip, turn over the agar slice and reposition the specimens. Replace a new cover slip with a drop of water onto the agar block and examine the specimens. After observation of the head, the agar block can be disassembled and the nematodes can be mounted onto an agar pad or other technique to observe the remainder of the body.

5.6 Staining Nematode Structures and Secretions

Morphological structures and nematode secretions can be stained with several different dyes provided that the mount is sealed with a sealer or nail polish (Premachandran *et al.*, 1988). Secretions from the amphids, secretory–excretory system and the phasmids were dependent upon the stain selected and the type of nematode. Also, particular morphological structures were stained with various types of dyes, including stylets, spicules and whole reproductive systems.

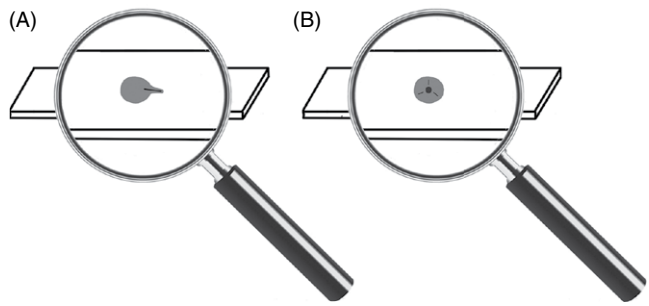

Fig. 5.8. Drawing showing how *en face* and cross-sections of nematodes are made from specimens infiltrated with glycerin. **(A)** A small piece of glycerin jelly is melted on a glass slide and a small dab is pulled away from the main portion with a dissecting needle or nematode pick. A nematode is placed in the narrow portion and the head is cut off with a scalpel. **(B)** The head is transferred with a pick to another small drop of glycerin that has been melted onto a glass slide. Three appropriately sized pieces of glass wool are put into the jelly and the head is mounted in the centre with the top facing upward. A cover slip is placed on the jelly and gently pushed down into the glycerin until it is supported by the three glass rods. A permanent marking pen is used to mark the bottom of the slide to point to the location of the specimen in the jelly. (After Basir, 1949.)

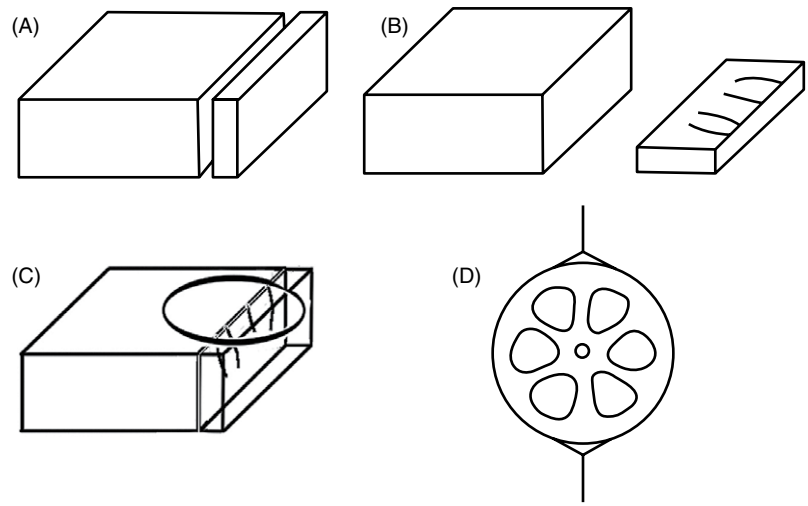

Fig. 5.9. Drawing showing how *en face* views are made of nematodes that are fixed in formalin or glutaraldehyde. **(A)** A 3 mm thick layer of 1.7% water agar is poured in a Petri dish and allowed to solidify. Using a razor blade, a 12 mm square is cut from the agar and placed on a glass slide, and a 2 mm slice is cut from one end of the block. **(B)** Three or four specimens that have been fixed in formalin or glutaraldehyde for at least 24 h are placed on the exposed surface of the cut agar with the anterior end near the edge of the block. **(C)** The block is returned to its upright position and location on the larger block. A small drop of tap water is placed on a cover slip that is inverted and placed on top of the agar block, covering the anterior ends of the nematodes. **(D)** Observations are made with an oil-immersion objective. If the specimens are not properly oriented, the mount can be taken apart and the nematodes can be rearranged. At the end of the observations, the specimens can be removed from the block and mounted on an agar pad for additional studies. (After Esser, 1986.)

5.6.1 Vital stains

Distinguishing between live and dead nematodes can be achieved with stains that colour either the live or the dead nematode, depending upon the stain. Potassium permanganate has been shown to stain the body contents of several nematode species a light amber to deep brown (Jatala, 1975). Unfortunately, this stain rapidly kills specimens of *Trichodorus,* so that the concentration and length of exposure varies for different nematodes. Therefore, nematodes that are to be evaluated for vitality have to be exposed to a known concentration for a specific time period in order to be reliable. Other stains that may be useful for distinguishing live from dead nematodes include New Blue R (Shepherd, 1962), Meldola Blue and Nile Blue A (Ogiga and Estey, 1974), and Eosin-y (Chaudhuri *et al.*, 1966). Enzymatically induced fluorescence may also distinguish live and dead nematodes (Bird, 1979; Forge and MacGuidwin, 1989). For further details of vital/non-vital stains see Perry, Chapter 9, this volume.

5.7 Staining Nematodes in Plant Material

5.7.1 Staining endoparasitic nematodes in roots

Nematodes that are endoparasites of plant tissues can be revealed inside of plant tissue by staining them to make them visible. Several techniques that have been used in the past, including the McBryde method and the lactophenol method, are problematic because McBryde uses the highly regulated chloral hydrate and the other uses the highly toxic lactophenol (refer to health and safety regulations before use). Clearing the roots with sodium hypochlorite (Byrd *et al.*, 1983) is much safer; even though it is a rapid oxidant,

it can be safely used with proper precautions, and it shortens the staining procedure and eliminates destaining of the root tissue.

- Gently wash the soil from the roots in a bucket of water.
- Cut large root systems into small segments.
- Place roots into a small beaker and add 100 ml of tap water and chlorine bleach (5.25% NaOCl) as follows:
  - 20% solution for young, tender roots;
  - 40% solution for older, harder roots; and
  - 60% solution for more lignified and mature roots.
- Soak for 4 min.
- Rinse in running tap water for 45 s and soak in water for 15 min.
- Drain, add 50 ml of tap water, and 1 ml of stock acid fuchsin stain (3.5 g acid fuchsin in 250 ml acetic acid, and 750 ml of tap water).
- Heat for 30 s in a microwave oven or on a hot plate.
- Cool, drain, and rinse the roots in tap water.
- Transfer the roots to 30 ml of acidified glycerin (3–5 drops of 5 N HCl) and destain in a microwave for 30 s.
- Store in acidified glycerin and observe the roots in the lid of a Petri dish a small amount of glycerin and covered with the bottom of the dish or with a small number of roots and glycerin pressed between two glass slides.

5.7.2 Staining egg masses of root-knot nematodes

Egg masses of root-knot nematodes are more easily counted by staining them with the dye. The most commonly used method is with red Phloxine B; however, red food colouring may be safer and friendlier to the environment (Thies, 2002). Steps for staining with Phloxine B are as follows:

- Gently wash the soil from the roots in a bucket of water.
- Cut large root systems into small segments.
- Place roots into a small beaker and add 100 ml of an aqueous solution of Phloxine B (0.15 g l^{-1} of tap water) for 15–20 min.
- Rinse in tap water to remove the residual Phloxine B.
- Count the number of egg masses by visually examining the stained roots either with the naked eye or with a magnifying aid such as a hand lens, dissecting microscope, or other reading glasses.

5.7.3 Histology staining

Plastic embedding (Pijanowski *et al.*, 1972):

- Fix in 4% formaldehyde in 50 mM PIPES buffer (pH 6.9) for 2–10 days at 4°C.
- Dehydrate in an ethanol series (1 h each: 15, 30, 50% v/v), with gentle shaking, at 4°C or in ice.
- Incubate overnight at 4°C in 70% ethanol.
- Dehydrate in 85% ethanol and three times in 100% ethanol (1 h each) on ice.
- Replace ethanol by 50% ethanol-butyl-methylmethacrylate [BM, 4:1 containing 0.1 mM dithiothreitol (DDT)] at 4°C overnight (Kronenberger *et al.*, 1993).
- Replace by 100% methacrylate mixture containing 0.1 mM DTT, and keep overnight at 4°C.
- Replace with fresh BM embedding medium containing 0.5% benzoin ethyl ether (100% BM and BEE) for 1 h up to overnight (or longer).

- Place samples in plastic capsules containing 100% BM, 0.5% BEE and 0.1 mM DDT, and polymerize at 4°C for 6 h under UV light.
- After polymerization, samples can be kept indefinitely.

Sectioning methacrylate-embedded nematode material:

- Remove polymerized samples from the capsule and section to 5 µm (greater thickness may cause loss of sections from slides).
- Float sections on drops of sterile water on polylysine-coated glass slides.
- Dry on a hot plate at 60°C.
- Make sections stick to the slides by incubating overnight at 42°C.
- Screen slides for the best sections.
- Keep in dry slides boxes at room temperature until they are to be stained.

5.7.4 Staining techniques

Fix infected plant tissue for 24–48 h in 2% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2 for 48 h. Dehydrate the tissues in a stepped series of tertiary butyl alcohol, infiltrate with paraffin, section with a microtome, and mount the paraffin sections on glass slides according to Daykin and Hussey (1985). After the sections are mounted on slides, stain the tissues with one of the following procedures of choice: either the Johansen's quadruple stain, the Sass safranin fast green stain, or the Triarch quadruple stain.

Johansen's quadruple stain (Johansen, 1940):

xylene	5 min
xylene-absolute ethanol (1: 1)	5 min
95% ethanol	5 min
70% ethanol	5 min
safranin 0 solution[a]	6–24 h
rinse in tap water	
1% aqueous methyl violet 2B	10–15 min
rinse in tap water	
95% ethanol-methyl cello solve-tertiary butyl alcohol (1:1:1)	15 s
fast green FCF solution[b]	3 min
95% ethanol-tertiary butyl alcohol (1:1) plus 0.5% glacial acetic acid	15 s
orange G solution[c]	3 min
clove oil-methyl cellosolve-95% ethanol (1:1:1)	15 s
clove oil-absolute ethanol-xylene (1:1:1)	15 s
xylene	5 min
xylene	5 min or more

[a] Safranin O solution: dissolve 4 g safranin O in 200 ml of methyl cellosolve. When it is dissolved, add 100 ml of 95% ethanol and 100 ml of distilled water, 4 g sodium acetate, and 8 ml of formalin.
[b] Fast green FCF solution: add 0.25 g fast green FCF to 50 ml of a solution composed of methyl cellosolve and clove oil (1:1). After the fast green has dissolved, add 150 m1 of 95% ethanol, 150 ml of tertiary butyl alcohol, and 3.5 ml glacial acetic acid.
[c] Orange G solution: dissolve 1 g of orange G in 200 ml of methyl cellosolve and add 100 ml 95% ethanol.

Sass safranin and fast green stain (Sass, 1951):

xylene	5 min
absolute ethanol	5 min
95% ethanol	5 min
70% ethanol	5 min

50% ethanol	5 min
30% ethanol	5 min
1% aqueous safranin O	1–12 h
rinse in tap water	
30% ethanol	3 min
150% ethanol	3 min
70% ethanol	3 min
95% ethanol	3 min
0.1% fast green FCF in 95% ethanol	5–30 s
absolute ethanol	15 s
absolute ethanol	3 min
xylene : absolute ethanol (1:1)	5 min
xylene	5 min
xylene	5 min or more

Triarch quadruple stain (Berlyn and Miksche, 1976):

xylene	5 min
xylene	5 min
xylene : absolute ethanol (1:1)	5 min
95% ethanol	5 min
70% ethanol	5 min
1% safranin O in 50% ethanol	5–15 min
rinse in distilled water	
1% aqueous crystal violet	1–2 min
rinse in distilled water	
absolute ethanol	30 s
absolute ethanol	30 s
orange G[a] – fast green[b] (135 ml – 15 ml)	3 min
orange G – fast green (145 ml – 5 ml)	2 min
orange G – fast green (148 ml – 2 ml)	2 min
orange G	2 min
absolute ethanol	1 min
xylene	5 min
xylene	5 min or more

[a] Orange G: dissolve 0.4 g orange G in 100 ml clove oil.
[b] Fast green: dissolve 1 g fast green FCF in 100 ml absolute ethanol.

Handling, fixing, mounting, and staining nematodes requires much patience and careful attention to detail but these are essential techniques and must be mastered.

5.8 References

Baker, A.D. (1953) Rapid method for mounting nematodes in glycerine. *The Canadian Entomologist* 85, 77–78. DOI: 10.4039/Ent8577-2.

Basir, K.M.A. (1949) An easy method for the preparation of *en face* view of small nematodes. *Transactions of the American Microscopical Society* 68, 123–126. DOI: 10.2307/3223261.

Berlyn, G.P. and Miksche, J.M. (1976) *Botanical Microtechnique and Cytochemistry*. Iowa State Univ. Press, Ames, Iowa, USA.

Bird, A.F. (1979) A method of distinguishing between living and dead nematodes by enzymatically induced fluorescence. *Journal of Nematology* 11, 103–105.

Boag, B. (1982) Effect of different concentrations of formalin and on the fixation of *Longidorus elongatus*. *Nematologia Mediterranea* 10, 115–118.
Brunel Microscopes Secure (n.d.) Available at: http://www.brunelmicroscopessecure.co.uk/acatalog/universal.html (accessed 25 February 2019).
Byrd Jr, D.W., Kirkpatrick, T. and Barker, K.R. (1983) An improved technique for clearing and staining plant tissue for detection of nematodes. *Journal of Nematology* 14, 142–143.
Chaudhuri, E., Dick, R.S. and Engelbrecht, H.A. (1966) Staining of free-living nematodes by Eosin-y dye. *Nematologica* 12, 337–344. DOI:10.1163/187529266X00761.
Chitwood, B.G. and Wehr, E.E. (1934) The value of cephalic structures as characters in nematode classification with special reference to the super family Spiruroidea. *Zeitschrift für Parasitenkunde* 7, 273–335.
Cobb, N.A. (1920) Micro-technique, Sec. III. To obtain an end view of a nema, rotifer, or other similar small object. *Transactions of the American Microscopical Society* 39, 236–237.
Coyne, D.L., Nicol, J.M. and Claudius-Cole, B. (2007) *Practical Plant Nematology: a Field and Laboratory Guide*. SP-IPM Secretariat, International Institute of Tropical Agriculture (IITA), Cotonou, Benin.
Curran J. and Hominick, W.M. (1981) Effects of mounting methods on taxonomic characters of adult male mermithids (Nematoda: Mermithidae). *Nematologica* 2 (1980), 455–446. DOI: 10.1163/187529280X00387.
Daykin, M.E. and Hussey, R.S. (1985) Staining and histo-pathological techniques in nematology. In: Barker, K.R., Carter, C.C. and Sasser, J.N. (eds). *An Advanced Treatise on* Meloidogyne: *Volume II*. North Carolina State University Graphics, Raleigh, North Carolina, USA, pp. 39–48.
De Grisse, A.T. (1969) Redescription ou modification de quelques techniques utilizes dans l'étude des nematodes phytoparasitaires. *Verslagen en Mededelingen van de Plantenziektenkundige Dienst, Ghent* 34, 351–369.
De Ley, P., Blaxter, M. and Giuliano, D. (1995) Extracting, fixing and mounting nematodes. WORMWOOD Home Page (A resource for nematode phylogeny). Available at: http://xyala.cap.ed.ac.uk/research/nematodes/fgn/worm/extrafix.html (accessed 20 July 2020).
Eisenback, J. D. (1985) Techniques for preparing nematodes for scanning electron microscopy. In: Barker, K.R., Carter, C.C. and Sasser, J.N. (eds). *An Advanced Treatise on* Meloidogyne, *Vol. 2, Methodology*. North Carolina State University Graphics: Raleigh, North Carolina, USA, pp 79–105.
Eisenback, J.D. (2012) A technique for making high-resolution megapixel mosaic photomicrographs of nematodes. *Journal of Nematology* 44, 260–263.
Eisenback, J.D. (2015) Resources for nematologists. *Nematology Newsletter* 61, 16.
Esser, R.P. (1973) A four-minute lactophenol fixation method for nematodes. *Plant Disease Reporter* 57, 1045–1046.
Esser, R.P. (1974) Two permanent mounting methods compared after six years. *The Helminthological Society of Washington* 41, 10–13.
Esser, R.P. (1986) A water agar *en face* technique. *The Helminthological Society of Washington* 53, 254–255.
Fagerholm, H.P. (1979) Nematode length and preservatives, with a method for determining the length of live specimens. *Journal of Parasitology* 65, 334–335.
Forge, T.A. and MacGuidwin, A.E. (1989) Nematode autofluorescence and its use as an indicator of viability. *Journal of Nematology* 21, 399–403.
Goodey, J.B. (1963) *Laboratory Methods for Work with Plant and Soil Nematodes*. Technical Bulletin, Ministry of Agriculture, Fisheries and Food, No. 2, 4th edn. Ministry of Agriculture, Fisheries and Food, London, UK.
Grewal, P.S., Richardson, P.N. and Wright, D.J. (1990) Effects of killing, fixing and mounting methods on taxonomic characters of parthenogenetic adult female *Caenorhabditis elegans* (Nematoda: Rhabditidae). *Revue de Nématologie* 13, 437–444.
Hooper, D.J. (1986) Handling, fixing, staining and mounting nematodes. In: Southey, J.F. (ed.) *Laboratory Methods for Work with Plant and Soil Nematodes* 6th edn. Her Majesty's Stationery Office, London, UK, pp. 59–80.
Jatala, P. (1975) Efficacy of potassium permanganate in differentiating between live and dead nematodes. *Annals of Applied Biology* 80, 109–113. DOI: 10.1111/j.1744-7348.1975.tb01605.x.
Johansen, D.A. (1940) *Plant Microtechnique*. McGraw-Hill Book Co., New York, New York, USA.
Kleynhans, K.P.N. (1999) *Collecting and Preserving Nematodes. A Manual for Nematology SAFRINET, the Southern African (SADC) LOOP of BioNET-INTERNATIONAL*, National Collection of Nematodes Biosystematics Division ARC – Plant Protection Research Institute Pretoria, South Africa.
Kronenberger, J., Desprez, T., Höfte, H., Caboche, M. and Traas, J. (1993) A methacrylate embedding procedure developed for immunolocalization on plant tissues is also compatible with *in situ* hybridization. *Cell Biology International* 17, 1013–1021. DOI: 10.1006/cbir.1993.1031.
Lamberti, F. and Sher, S.A. (1969) A comparison of preparation techniques in taxonomic studies on *Longidorus africanus* Merry. *Journal of Nematology* 1, 193–200.

Ogiga, I.R. and Estey, R.H. (1974) The use of Meldola blue and Nile blue A for distinguishing dead from living nematodes. *Nematologica* 20, 271–276. DOI: 10.1163/187529274X00302.
Pest CABweb. (2002). Available at: http://cabweb.org/Identification/lipprep2.htm (accessed 28 August 2018). CAB International. Wallingford, UK.
Pijanowski, G.J., Cleveland, D.J. and Georgi, J.R. (1972) A glycol-methacrylate embedding technique for nematode whole mounts. *Cornell Veterinarian* 62, 333–336.
Premachandran, D., von Mende, N., Hussey, R.S. and McClure, M.A. (1988) A method for staining nematode secretions and structures. *Journal of Nematology* 20, 70–78.
Ravichandra, N.G. (2010) *Methods and Techniques in Plant Nematology*. PHI Learning Private Limited, New Delhi, India.
Ryss, A.Y. (2003). Express technique to prepare permanent collections slides of nematodes. *Zoosystematica Rossica* 11 2002, 257–260.
Ryss, A.Y. (2017) A simple express technique to process nematodes for collection slide mounts. *Journal of Nematology* 49, 27–32.
Sass, J.E. (1951) *Botanical Microtechnique*. Iowa State College Press, Ames, Iowa, USA.
Seinhorst, J.W. (1959) A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. *Nematologica* 4, 67–69. DOI: 10.1163/187529259X00381.
Seinhorst, J.W. (1962) On the killing, fixing and transferring to glycerin of nematodes. *Nematologica* 8, 29–32. DOI: 10.1163/187529262X00981.
Seinhorst, J.W. (1966) Killing nematodes for taxonomic study with hot F.A. 4:1. *Nematologica* 12, 178. DOI: 10.1163/187529266X00239
Shepherd, A.M. (1962) New blue R. a stain that differentiates between living and dead nematodes. *Nematologica* 8, 201–208. DOI:10.1163/187529262X00431.
Southey, J.F. (ed.) (1986) *Laboratory Methods for Work with Plant and Soil Nematodes* 6th edn. Ministry of Agriculture, Fisheries and Food, London, UK.
Stone, A.R. (1971) Effect of processing on measurements of *Heterodera rostochiensis* larvae. *Nematologica* 17, 167–171.
Stynes, B.A. and Bird, A.F. (1981) Effects of methods of killing, killing and mounting on measurements of *Anguina agrostis*. *Nematologica* 26 (1980), 467–474. DOI: 10.1163/187529280X00396.
Sulston, J. and Hodgkin, J. (1988) Methods. In: Wood, W.B. (ed.) *The Nematode* Caenorhabditis elegans: *Cold Spring Harbor Laboratory*, Cold Spring Harbor, Massachusetts, USA, pp. 581–606.
Thies, J.A. (2002) Red food coloring stain: improved, safer procedure for staining nematodes in roots and egg masses on root surfaces. *Journal of Nematology* 34, 179–181.
Thorne, G. (1961). *Principles of Nematology*. McGraw-Hill Book Company, Inc., New York, New York, USA.
van Bezooijen, J. (2006) *Methods and Techniques for Nematology*. Available at: http://nematologia.com.br/wp-content/uploads/2014/03/vanBezo.pdf (accessed 28 August 2018).
Yoder, M., King, I.W., Tandingan De Ley, I., Mann, J., Mundo-Ocampo, M., Poiras, L., De Ley, P. and Blaxter, M. (2006) DESS: a versatile solution for preserving morphology and extractable DNA of nematodes. *Nematology* 8, 367–376. DOI: 10.1163/156854106778493448.
Zuckerman, B.M., Mai, W.F. and Harrison, M.B. (1985) *Plant Nematology Laboratory Manual*. The University of Massachusetts Agricultural Experiment Station, Amherst, Massachusetts, USA.

6 Culturing Techniques

ROSA H. MANZANILLA-LÓPEZ[1] AND RALF-UDO EHLERS[2]

[1]*Instituto Politécnico Nacional, Centro de Productos Bióticos, Yautepec, Mexico;*
[2]*e-nema GmbH, Schwentinental, Germany*

6.1 Introduction

A nematological culture is a method of multiplying nematodes by enabling them to reproduce and increase in a culture medium or a specific host under controlled conditions. However, although under culture conditions some plant-parasitic nematodes can develop beyond the second-stage juvenile, they do not reproduce (De Ley and Mundo-Ocampo, 2004). In this chapter we have revised and updated information in Hooper (1986), but focused mainly on plant-parasitic nematodes and entomopathogenic nematodes, with some mention of soil free-living nematodes.

Axenic culture of *Caenorhabditis elegans* and other nematodes has been described by Vanfleteren (1978) and Wormbook is an on-line source for culture techniques for *C. elegans* on solid and liquid media (Stiernagle, 2006). The methods described can be applied to many free-living rhabditid and diplogastrid nematodes. Most applications use *Escherichia coli* OP50 cultures to grow the nematodes. De Ley and Mundo-Ocampo (2004) covered a range of basic culture techniques for *C. elegans* that can be adapted to other bacteria-feeding nematodes. The same authors also covered the culture of free-living soil, fresh water, brackish and marine nematodes. Culturing techniques for brackish and marine nematodes are also given by Moens and Vincx (1998).

6.2 Collecting Nematodes to Start a Culture

In order to establish cultures of plant-parasitic nematodes, soil samples containing the target nematode species must be collected from plant rhizospheres in natural sites or agriculture fields, not forgetting the importance

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
DOI: 10.1079/9781786391759.0006

of an accurate identification of the plant. Collecting the plants, fruit or seed may help for plant identification, but also for initial maintenance of the sampled population in the glasshouse. If after extraction from soil or plant samples the nematodes are present in low numbers, inoculating pots with a potential host plant can help to build up nematode populations (De Ley and Mundo-Ocampo, 2004). Usually, glasshouse cultures are used to start nematode cultures under aseptic conditions (i.e. contamination free).

6.3 Axenization and Gnotobiology in *in Vitro* Cultures

Axenization is the process of isolating a particular organism from all others to be studied in a pure culture. Gnotobiology is 'the study of a single species in the absence of other demonstrable species or in the presence of only known species' (Zuckerman, 1971), and for plant-parasitic nematodes it has the advantage of requiring reduced growth chamber or incubator space and non-daily maintenance in comparison with glasshouse cultures. Gnotobiology is essential in any pathogenesis evaluation of plant-nematode interactions and application of Koch postulates to plant nematology (Mountain, 1960). Studies such as transcriptomics, proteomics or metabolomics require nematodes that are free of biological contamination and are easily obtainable in large quantities. *In vitro* cultures and the use of model host plants, such as *Arabidopsis thaliana*, can be maintained in controlled environment cabinets or incubators throughout the year (Díaz-Manzano *et al.*, 2016) and enable studies on host–parasite relationships. According to Zuckerman (1971) '**gnotobiotic cultures**' describes cultures where the number of types of organisms, but not necessarily the species, is known; '**monoxenic cultures**' contain one known associated organism; and '**axenic cultures**' have no associated organisms. The terms '**agnotobiotic**' or '**xenic**' cultures refer to the presence of an unknown number of associated organisms (usually a mixed microbial flora).

Successful *in vitro* production of nematodes in the numbers and physical condition needed for small or large experiments, either in the laboratory, glasshouse or field, will require an initial basic knowledge of: (i) the nematode species and host biology; (ii) nematode host culturing methods; and (iii) media composition to grow both nematode and host. According to the purity of their components, media are classified as **holidic**, **meridic** and **oligidic** (Dougherty, 1959). All components of holidic media have a chemically known composition, whereas meridic media contain one or more ingredients whose composition is unknown, and oligidic media contain impure organic compounds (Parra, 2012). Recipes for culture media by White (1943a,b), Tiner (1960) and Murashagi and Skoog (1962), for example, are commonly used in nematology laboratories to grow plant-parasitic nematodes axenically on root tissue from a variety of plants; several formulated media are commercially available. Myers was the first to report axenic cultures of a stylet-bearing nematode, *Aphelenchoides sacchari*, which reproduces rapidly in an oligidic medium containing liver extract and dextrose (Myers, 1967; Myers *et al.*, 1971). Vanfleteren (1980) reviewed various axenic media for continuous culture of *C. elegans* and various other nematodes.

Examples of monoxenic systems include nematode cultures in excised roots and seedlings of tomato (Orion *et al.*, 1980, 1995; Sudirman and Webster, 1995; Hutangura *et al.*, 1998), okra *Abelmoschus esculentus* (Tanda *et al.*, 1980), onion root cultures (Mitkowski and Abawi, 2002), and plant models such as *A. thaliana* (Sijmons *et al.*, 1991) and *Lotus japonicus* (Lohar and Bird, 2003; Cabrera Poch *et al.*, 2007; Amin *et al.*, 2014). In order to ensure that only the target species of nematode is present in such systems, surface sterilization, most commonly of eggs and second-stage juveniles (J2), is necessary. A combination of several disinfection methods can be used but excessive or insufficient disinfectant doses can affect nematode survival or allow subsequent culture contamination (Díaz-Manzano *et al.*, 2016).

For *C. elegans*, Dougherty (1959) recommended about 50 gravid females be pipetted into a small 'boat' of fine wire-gauze placed in a dish half full of 10 volume H_2O_2. The boat is removed after 10 min and the dead females rinsed by gently raising and lowering the boat in three successive dishes of sterile water. It is then placed in the mouth of a centrifuge tube almost full of Ringer's solution plus of streptomycin and penicillin (100 U ml^{-1}). After 24–48 h, juveniles that have hatched and escaped from the dead females pass through the wire mesh and can be concentrated by gentle centrifugation. Patel and McFadden (1978) suspended and stirred gravid females in 0.4 M NaOH for 3 h at 25°C. The alkali partly digested the nematodes and served as a sterilant. The eggs released are centrifuged in a sucrose gradient (58,800 *g* for 3 h) and eggs from the 1.13 density band were thoroughly washed in dH_2O, re-suspended in 0.4 M NaOH, then washed

repeatedly with sterile water. Jatala *et al.* (1974) surface-sterilized eggs and adults of *Pristionchus lheritieri* with 0.1% $HgCl_2$ for 3 min. Chantanao and Jensen (1969) described a method for exchange of the indigenous bacterial flora of *P. lheritieri* for plant-parasitic bacteria in their progeny. Anderson and Coleman (1977) also axenized *P. lheritieri* using 0.1% merthiolate followed by treatment with antibiotics on an agar plate. Some bacteriophagous nematodes can be axenized in antibiotic solutions. Cryan (1963) used a combination of penicillin, streptomycin and nystatin to axenize *Rhabditis pellio*, *Caenorhabditis briggsae* and *Panagrellus redivivus*.

6.4 Cleaning and Decontamination of Plant-parasitic Nematodes

Plant-parasitic nematodes are commonly extracted from soil, cysts or egg masses and will need to be cleaned and decontaminated before starting a culture. Decontamination includes two distinct processes: disinfection and sterilization. Disinfection destroys pathogenic microorganisms and removes most organisms present on surfaces, whilst sterilization refers to any process that eliminates or kills all forms of life. Disinfection is generally achieved by passing nematodes through one or more solutions of antimicrobial agents such as disinfectants or antiseptics. Sterilization may require the use of more than one physical and chemical treatment as well as the biological or synthetic substances used to kill bacteria (antibiotics) and fungi (antimycotics); the choice of protocols must be based on nematode species and culture. Cleaning and disinfecting nematodes can be appropriate for pot culturing, but *in vitro* culturing will also require nematodes to be treated with a sterilizing agent (sterilant).

Sterilization of nematodes has focused mainly on surface sterilization and testing for contaminants, of which fungi and bacteria have been the main targets as, for a long time, most plant-parasitic nematodes were considered not to bear microorganisms in their digestive tracts (Zuckerman, 1971; Hooper, 1986). However, despite surface sterilization of *R. similis* with 0.1% benzalkonium chloride (Haegeman *et al.*, 2009) and *Pratylenchus* spp. with 0.01% streptomycin sulfate (Denver *et al.*, 2016), some microorganisms have been found inside the nematodes as endosymbiont-like organisms whose function is largely unknown. *Wolbachia pipientis* (α-proteobacteria) occurs in *R. similis* (Haegeman *et al.*, 2009) and *W. pipientis* and *Cardinium hertigii* (Bacteroidetes) in *P. penetrans* (Brown *et al.*, 2016, 2018; Denver *et al.*, 2016). *Xiphinema americanum* has one or more obligate endosymbionts with nutrient supplementation roles (Brown *et al.*, 2015; Palomares-Rius *et al.*, 2016; Howe *et al.*, 2019). Transmission electron microscopy (TEM) and light microscopy have shown a *Cardinium*-like endosymbiont in *Globodera rostochiensis* (Shepherd *et al.*, 1973; Walsh *et al.*, 1983a,b), *Heterodera avenae* (Yang *et al.*, 2017), *H. goettingiana* (Shepherd *et al.*, 1973) and *H. glycines* (Endo, 1979; Noel and Atibalentja, 2006). Although antibiotics have the ability to destroy bacteria within the body, antibiotic treatment (e.g. doxyclycine or rifampicin) of the *Wolbachia* endosymbiont of animal-parasitic filarial nematodes has not produced totally 'cured' yet viable filarial worms (Slatko *et al.*, 2010). In future, production of axenic cultures (see Section 6.5) should consider the existence of endosymbionts and the fact that surface sterilization may not always deliver nematodes free of biological contaminants.

6.4.1 Cleaning of nematodes in water

Axenized nematodes will need to be cleaned and/or surface sterilized before being added to cultures; it is a good practice to wash nematodes to remove soil or root debris following exposure to any chemical sterilant. Individual cleaning of nematodes can be performed, under the microscope, by gently rubbing the nematode's body with a short piece of hair or plastic bristle glued to a handling needle while nematodes are kept immersed in water in a suitable glass container (e.g. watch glass, Syracuse dish, Petri dish). Cleaning of nematodes can be performed by several rinses in sterile tap or distilled water (dH_2O) contained in sterile laboratory ware. Small numbers of nematodes can be cleaned by using a handling needle to pass them through successive drops of sterile water contained in sterilized excavated glass slides (Hooper, 1986). Large numbers of nematodes can be washed on a sieve (mesh aperture 50 µm or less) using several changes of sterile dH_2O. Alternatively, an individual sieve containing the nematodes is partially submerged into a sterile glass, flat-bottomed evaporating dish containing the sterile water (Fig. 6.1A). The latter can be replaced with fresh

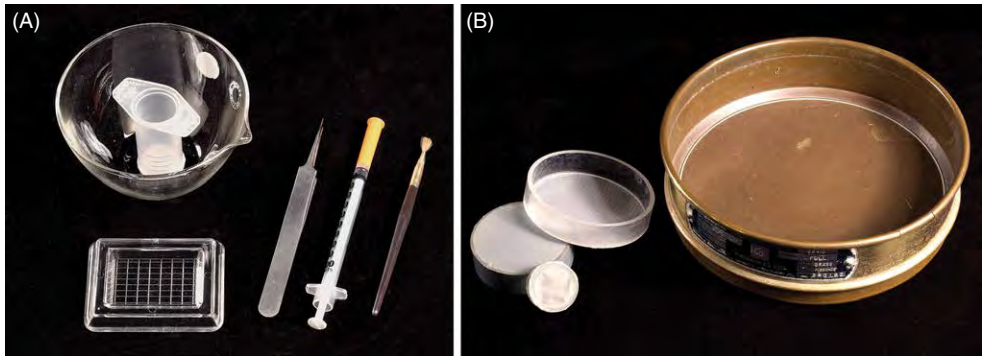

Fig. 6.1. Laboratory ware and handling tools for washing nematodes. **(A)** From left to right: flat-bottomed evaporating dish, small sieve, brush, counting dish. **(B)** Small sieves made from PVC tubing to which a fine nylon mesh has been glued to one end. (A, B: Copyright Rothamsted Research Ltd.)

sterile water after nematodes are given a first rinse and this process can be repeated several times (at least three times). Small sieves can be purchased or sieves can be made from PVC tubing to which a fine nylon mesh (5 or 10 µm aperture) has been glued to one end (Fig. 6.1B). These sieves can be cleaned by plunging them into boiling water or they can be sprayed with 70% alcohol, dried and treated with ultra-violet (UV) light (20 min) in a flow cabinet. Large number of nematodes in water suspension can be cleaned by centrifuging or be allowed to settle to the bottom of a tube or other sterile glass container and the supernatant carefully decanted prior to adding fresh sterile water or a sterilant followed by shaking or vortexing to re-suspend them; the process is repeated three to four times. Sterile flat-bottomed evaporating dishes can be very useful for washing, rinsing in sterile dH_2O and decanting the nematodes.

6.4.2 Surface sterilization

There are different methods to surface sterilize different life stages of nematodes. Surface sterilization can be carried out by using sterilants alone or in combination with antibiotics. However, any chosen method should be tested to assess its efficacy on any nematode species, as sensitivity of species and life cycle stages to chemicals differs (Ko *et al.*, 1996). Surface chemical sterilization can cause high nematode mortality, thus requiring a large number of starting specimens, and methods have been developed to treat a small number of axenized nematodes without the potential deleterious effects of chemicals and, at the same time, to be applicable to most nematode species (Ko *et al.*, 1996).

Some of the commonest chemical surface sterilants have been phased out and others, such as methoxyethyl mercury chloride (Aretan) and merthiolate, although effective and still in use, are highly hazardous and should be used in compliance with health and safety regulations. Sterilants such as $HgCl_2$ must be used and disposed of appropriately, again according to health and safety regulations. The latter recommendation also applied to antibiotics including those added to culture media. Cetrimide is commercially available as Cetavlon® but other sterilants are neither easy to obtain nor easy to dispose properly.

It is of utmost importance to work always under aseptic conditions, preferably in a flow cabinet, and all handling tools and laboratory ware should be sterilized and, together with the microscope, be exposed to UV light before use. Check for sterility of the water as some bacteria can tolerate autoclaving temperatures.

6.4.2.1 Inorganic disinfectants

One of the cheapest and most common inorganic disinfectants is commercial sodium hypochlorite (NaOCl; bleach), which also is used to dissolve the gelatinous matrix of root-knot nematodes where eggs are embedded. To obtain gelatinous matrix-free eggs of root-knot nematodes using 'Purex' (= 10% NaOCl), prepare a 5.25% NaOCl solution and submerge the egg mass in the solution for 4 min, then rinse with a sterile

water wash (Loewenberg *et al.*, 1960). Mendy *et al.* (2017) surface-sterilized eggs in a 10% NaOCl solution for 3 min followed by several washes with sterile water. The egg suspension was further washed with 150 µl nystatin (10,000 U ml^{-1}) and 2 ml gentamycin sulfate (22.5 mg ml^{-1}) in a total volume of 30 ml. The suspension was stored at room temperature in darkness. Freshly hatched J2 were rinsed in water, incubated for 20 min in 0.5% (w/v) streptomycin-penicillin and 0.1% (w/v) ampicillin-gentamycin solution, 3 min in 0.1% (v/v) chlorhexidine and washed three times with sterile autoclaved water.

To surface sterilize cysts and J2 (Eisenback and Zunke, 1998), place mature cysts filled with embryonated eggs into an excavated glass block containing 0.5% NaOCl; leave for 1 min, then replenish the bleach leaving the cysts to soak for another minute. Repeat the exchange of solutions and soak the cysts for 3 min. Cysts (10–20) can then be transferred directly on the agar containing the excised plant roots.

6.4.2.2 Surface sterilization and decontamination using agar

A reliable method for initial axenization and surface decontamination of nematodes is to allow them to crawl through water containing disinfectants (Goodman and Chen, 1967) or water agar (Chen, 1964). Huang and Becker (1997, 1999) surface decontaminated *Belonolaimus longicaudatus* in 1% agar. After extraction from bermudagrass (*Cynodon dactylon*) glasshouse culture, nematodes were hand-picked into sterile osmosis water (i.e. water purified by reverse osmosis) in a 3.5 cm diam. Petri dish. Under aseptic conditions, adult nematodes were hand-picked and embedded with the help of a needle in the centre of a 10 cm Petri dish containing 1% agar; dishes were incubated overnight in darkness at 26–27°C. The nematodes were considered surface decontaminated after migrating through the agar for at least 2 cm. Nematodes were then aseptically transferred onto nutrient agar (beef extract 3 g, peptone 5 g, agar 15 g, dH_2O 1 l, pH 6) in tri-Petri dishes with one nematode in each section, and incubated in darkness for 24 h. Nematodes in Petri dish sections in which no bacterial or fungal colonies appeared were used to inoculate excised root cultures with 60 females and 40 males per dish; cultures were sealed with Parafilm® and maintained in darkness at 26–27°C. Nematode eggs from the *in vitro* culture were transferred onto 1% water agar plated and incubated at 28°C overnight to obtain J2 that were inoculated onto 5-day-old corn (*Zea mays* 'Golden Jubilee') root cultures (Huang and Becker, 1999).

Moody *et al.* (1973) described a method for axenization of *Pratylenchus* spp. on agar:

- Concentrate nematodes in 2 ml of water.
- Prepare and autoclave 250 ml of 1% water agar and cool to 48–50°C before adding the nematode suspension to this agar.
- Swirl the nematode-agar suspension, pour 3 ml into the centre of sterile Petri dishes and allow the agar to solidify.
- Prepare and autoclave 1 l of 1% water agar, cool to 50°C, add 0.13 g Aretan and 6 g dihydrostreptomycin sulfate, and agitate until the chemicals are dissolved.
- Pour this antibiotic medium gently over the solidified nematode-agar suspension in each Petri dish to a depth of 5 mm.
- After 36 h incubation at 23°C nematodes will have made their way up through the antibiotic medium to the surface. Wash nematodes into a 100 ml beaker using 10 ml of sterile water per Petri dish.

6.4.2.3 Surface sterilization assisted by glass beads and glass fibre

One glass chromatography column (2.2 × 49 cm) is filled with a mixture made of 3 mm and 6 mm diam. glass beads supported by a platform of glass fibre, an antibiotic mixture (0.5% gentamycin, 0.5% tetracycline/oxytetracycline HCl, 0.0005% chlorhexydine digluconate in tap water) and fitted with a Nalgene® stopcock. Nematodes are passed through the glass column and surface-sterilized nematodes are aseptically rinsed free of sterilant (Krusberg and Sardanelli, 1984; Kaplan and Davis, 1990).

6.4.2.4 Surface sterilization using Pluronic F127 gel

Pluronic® F127 gel is a co-polymer gel of propylene oxide and ethylene oxide (polyglycol) that can inhibit the growth of many bacteria, fungi and actinomycetes, without affecting the growth of selected nematode species of *Meloidogyne*, *Pratylenchus*, *Radopholus* and *Rotylenchulus* (Ko and Van Gundy, 1988). Pluronic gel has the advantage that nematodes, plant tissues, secretory–excretory products and labile biologically active factors associated with the culture can be non-destructively extracted by liquefying the polyol-base medium. Pluronic gel can be used to examine aspects of nematode biology and host–parasite relationships (see Perry, Chapter 9, this volume). Encapsulation of the nematodes in the polyglycol at 20% (w/w) ensures retardation of growth of associated microbial contaminants acting as a cleansing agent.

Surface sterilization of nematodes using Pluronic gel F127 requires *ca* 50 nematodes to start an aseptic population but demands timely preparation of the tissue plates (Ko *et al*., 1996). Nematodes are individually picked and placed in a drop (25–50 µl) of 20% (w/v) Pluronic F127 polyol on an ice-cold circular sterile cover slip (12 mm, 0.13–0.17–mm thick) and left to gel at 25 or 30°C for 15 min. Each cover slip is then inverted and transferred to a distal point in a Petri plate baited with two to four pieces of sterile tomato root explants or alfalfa callus tissues. The Petri dish contains either agar or Gelrite®. Gelrite is a naturally derived gelling polymer produced by microbial fermentation that can be used in a variety of applications as a solidification agent instead of agar. The drop containing the nematodes is placed in contact with the agar or Gelrite surface; if needed, antibiotics or fungicides may be incorporated into the polyol drop. Plates are then incubated at 25°C. The agar or Gelrite portion is removed as soon as nematode tracks appear around the baits.

Plates are incubated to allow the nematodes to multiply. Alternatively, the nematode-infected callus tissues may be transferred to a new plate containing fresh callus tissues. Plates should be observed regularly for contamination; nematodes (adults, juveniles or eggs) can be extracted after 4 months from the callus tissues, root explants or the support base (agar or Gelrite) by the Baermann funnel method. Samples of nematodes and host tissues from plates can be tested for sterility by passage through nutrient glucose peptone, and potato dextrose agar (PDA).

6.4.3 Antiseptics and disinfectants for Tylenchida and Aphelenchida

6.4.3.1 Hibitane diacetate

Hibitane diacetate (synonym: chorhexidine acetate) is a bacteriocidal and bacteriostatic antiseptic. Axenic nematodes and *Meloidogyne* eggs were obtained by using 0.05% hibitane diacetate (bis (p-chlorophenyldiguanido) hexane diacetate) followed by rinsing with sterile water (Zuckerman and Brzeski, 1966; Paracer and Zuckerman, 1967). Roman and Hirschmann (1969) surface-sterilized *Pratylenchus* spp. with 0.1% hibitane diacetate for 18 min and rinsed for 2–3 min in each five changes of sterile dH_2O before inoculating the nematodes on alfalfa callus tissue.

6.4.3.2 Cetavlon

Cetrimide, brand name Cetavlon®, is a mixture of different quaternary ammonium salts used as an antiseptic. *Meloidogyne* J2 from egg masses were washed for 5 min in 0.1% Cetavlon, rinsed in sterile water, then immersed in 0.5% hibitane diacetate for 15 min and then rinsed in sterile distilled water (Peacock, 1959).

6.4.3.3 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a common surface disinfectant that can be used in combination with other disinfectants. To surface disinfect *Meloidogyne javanica* eggs, the egg masses are placed in hydrogen peroxide (3%) for 20 min, followed by sterile water washes (two or three), Cetavlon (0.1%) for 5 min, sterile water wash, hibitane diacetate (0.5%) for 5 min, then sterile water wash (Zuckerman, 1971).

6.4.3.4 Antibiotics

Several antibiotics are known to give good results in surface disinfecting nematodes; however, protocols and the list of appropriate antibiotics varies according to nematode species. Most protocols include more than one antibiotic, but one may be sufficient. Mountain (1955) placed single females of *P. penetrans* in successive drops of sterile water, then into 0.1% streptomycin sulfate for at least 15 min, followed by further rinsing in sterile water.

Combining antibiotics with antimycotics and commercial antibiotic-antimycotic products is useful. Cell Culture Gard (AppliChem ITW Reagents) (100× solution) is described as a combination of novel antibiotics also used in cell cultures that replaces penicillin, streptomycin, nystatin and amphotericin B. The composition of Merck A5955 (100× solution) includes 10,000 penicillin units, 10 mg streptomycin and 25 µg amphotericin B ml^{-1} and is suitable for nematode culture. A5955 has been used to surface disinfect *Ditylenchus angustus*. Nematodes were obtained by tearing rice tissue and passed from tap water through about a 300 µm mesh, then collected on a 5 µm mesh sieve and cleaned by allowing them to migrate through a cellulose sponge filter into water on A5955 solution 1% (v/v) and transferred to dH_2O. The nematodes were further surface sterilized in a solution of malachite green (0.1% w/v) for 15 min and then transferred to sterile dH_2O prior to inoculation on monoxenic cultures (Plowright and Akehurst, 1992).

An antibiotic has also been used in combination with Cetavlon and hibitane. Sawhney and Webster (1975) treated *Meloidogyne* egg masses sequentially with 0.1% penicillin then 0.1% streptomycin sulfate for 45 min each, then 0.05% Cetavlon for 1 min and 0.4% hibitane for 6 min, followed by rinsing in sterile water. Second-stage juveniles that hatched from the egg masses were treated with 0.3% hibitane for 15 min and then rinsed in sterile water.

Antibiotics have been combined with the histological stain malachite green. Krusberg (1961) passed *D. dipsaci*, *A. ritzemabosi* and *Pratylenchus* spp. through several (five or six) changes of a solution of 20 ppm malachite green plus 0.1% streptomycin sulfate in small Syracuse dishes; the nematodes were in contact with these solutions for about 4 h. *Aphelenchus*, *Aphelenchoides*, *Pratylenchus* and *Ditylenchus* spp. axenic specimens were obtained after 15 min immersion in 0.1% malachite green and then rinsed with sterile water (Hooper, 1986; Plowright and Akehurst, 1992). Treatments of 0.1% malachite green alone (15 min) or with 0.5% streptomycin sulfate were efficient to surface sterilize *P. penetrans* and did not significantly reduce nematode movement, nor attraction to and penetration into *Rosa dumetorum* 'Laxa' seedlings (Peng and Moens, 1999).

6.4.3.5 Mercury chloride

Mercury chloride (or mercuric chloride; $HgCl_2$) is highly toxic to humans. It is a broad-spectrum disinfectant and strong sterilant. Fenwick (1956) obtained viable axenic J2 from *Globodera rostochiensis* eggs treated with a 20 volume solution of $HgCl_2$ for 8 h followed by two washes in sterile water. Whitney and Doney (1970) hatched J2 of *H. schachtii* by incubating the cysts for 3–5 days in 4 mM zinc chloride, 10 ppm ethoxyethyl mercury chloride (Aretan), 0.01% dioctyl sodium sulphosuccinate (Triton X-100 could be used instead), 1 mg ml^{-1} streptomycin sulfate and 1000 U ml^{-1} penicillin G potassium. For pure culture studies, the J2 were placed in the hatching solution plus neomycin sulfate for 7 days; J2 were washed once in sterile water and then disinfested for 72 h in 150 ml of hatching solution. Zinc chloride not only works as a hatching stimulant but retards growth of microorganisms.

Meloidogyne incognita J2 were surface disinfected by placing them first on 5 µm sieves (Cell-Trics® filters), washed with 10 ml sterilized tap water and treated with 0.02% $HgCl_2$ for 3 min, and then with 4000 ppm streptomycin sulfate for another 3 min before being incubated for 4 h in 5 ml 1× CellCultureGuard (antibiotic solution) on a rotary shaker at 150 rpm (Elhady *et al.*, 2018). Afterwards, nematodes were washed on a 5 µm sieve and incubated overnight in sterilized tap water; surface sterilization was checked by plating them on R2A (Merck) for bacterial growth and potato extract glucose agar for fungal growth.

Eggs of *Meloidogyne* and *Nacobbus* have been surface sterilized with $HgCl_2$ before inoculating onto plates containing roots:

- Place egg masses in a glass container (glass block) with dH_2O and remove debris with forceps.
- Remove *ca* half the volume of water and add a solution of 20% NaOCl; shake for a minimum of 30 s, but not longer than 2 min.

- Clean the eggs by catching the suspension on small sieves of 60 µm (debris and J2 are retained) and 30 µm aperture (eggs are retained).
- Wash and rinse the eggs from the 30 µm sieve with dH_2O and collect them in a flat-bottomed evaporating dish.
- Suck up a suspension of eggs (*ca* 10–12 ml) using a 20 ml syringe containing 3 ml of sterile dH_2O.
- Suck up the sterilizing solution (2 ml of 0.1% $HgCl_2$) into the same syringe; mix well in the syringe for exactly 4 min.
- To wash away the $HgCl_2$, attach syringe to an autoclaved filter containing a 5 µm Whatman® cellulose nitrate membrane (13 mm diam.) and squeeze out the solution with the eggs.
- Use another syringe to rinse (2–3×) gently with sterile dH_2O; after most of the water has washed through, briefly wait and then force the rest of water out and gently suck some air in to dry the eggs.
- With sterile fine forceps remove the filter and spread the eggs onto the agar; using a cool, sterile scalpel blade, gently score the surface of agar plate in tramlines, trying not to cut the roots.
- Seal the plate with Parafilm and place in growth room.

Myers *et al.* (1965) treated *R. similis* with 0.01% aqueous $HgCl_2$ for 2 min. Brown and Vessey (1985) surface soaked *R. similis* for 4 h in an aerated 125 ml aqueous solution containing 0.1 g Aretan and 1 g streptomycin sulfate. After aeration, nematodes were left to settle for 2 h, rinsed successively with aqueous mercuric chloride (0.01 g l^{-1}) and twice with sterile dH_2O (see also banana fruit callus culture). Verdejo-Lucas and Pinochet (1992) surface disinfected *R. similis*, *Pratylenchus* spp. and *Zygotylenchus guevarai* in 0.01% $HgCl_2$ and 1% streptomycin sulfate for 5 min, rinsing them afterwards in sterile water. Dolliver *et al.* (1962) immersed *A. ritzemabosi* for 2 min in each of 100 ppm $HgCl_2$ and 1% streptomycin sulfate; Sutherland (1967) and van der Walt and De Waele (1989) used 0.1% $HgCl_2$ to axenize *A. besseyi* and *D. destructor*, respectively. Hajihassani *et al.* (2017) first centrifuged *D. weischeri* and *D. dipsaci* individuals at 1500 *g* for 3–4 min. After discarding the supernatant nematodes were transferred to sterile 2 ml microcentrifuge tubes containing streptomycin sulfate (4000 mg l^{-1}) and left overnight. Nematodes were transferred to a 1000 mg l^{-1} $HgCl_2$ solution for 10 min at 4°C, then rinsed with sterile water three times and stored in water at 4°C until use. *Mesocriconema xenoplax* were disinfected by incubation in 2-methoxyethyl $HgCl_2$ (16 µg ml^{-1}) for 5 h. Ten to 30 nematodes were transferred individually from the disinfectant solution to sterile plant cultures. Subcultures were subsequently established by transferring agar blocks containing nematodes and roots to fresh media (Westcott and Hussey, 1992).

6.4.3.6 Disinfectants and sterilants for Dorylaimida and Diphtherophorida

Dorylaimids and diphtherophorids are difficult to surface sterilise and remain alive and infective; however, some protocols have avoided problems. Goodman and Chen (1967) disinfected large numbers of *Paratrichodorus minor* without affecting nematode movement or ability to feed using two methods. In one method, nematodes were suspended for 20 h in 1.5% agar containing 100 ppm methoxyethyl $HgCl_2$ and were retrieved from the agar surface in a small volume of sterile dH_2O. The second method allowed the nematodes to migrate through absorbent cotton in a Melpar-Tiner storage trap filled with 100 ppm Aretan. As an alternative to using $HgCl_2$, *P. minor* were surface sterilized using 0.5% hibitane diacetate diffused in water agar (Chen, 1964; Zuckerman and Brzeski, 1966) and a combination of three antibiotics (Chen *et al.*, 1965), although repeated trials were needed to obtain a few living nematodes. Das and Raski (1968) surface-sterilized *X. index* by immersion in 0.01% Aretan or 0.1% dihydrostreptomycin sulfate for 1 h without destroying the infectivity or viruliferous capability of the nematode. Bird *et al.* (1968) used a mixture of 100 ppm (0.01%) streptomycin sulfate, 30 ppm (0.003%) aureomycin and 35 U ml^{-1} mycostatin followed by washing in sterile water to treat *P. minor*. Surface sterilization was accomplished by Bavaresco and Walker (1994) using 150 ml of 250,000 U l^{-1} penicillin + 250 mg l^{-1} streptomycin + 0.625 mg l^{-1} amphotericin B (Merck) solution to wash the nematodes as they settled through a Baermann funnel. After 3 h in this solution, the nematodes were rinsed three times with filtered sterile tap water and transferred to Petri dishes containing *Vitis rupestris* roots.

6.4.4 Testing for contamination by microorganisms after surface sterilization

Dougherty *et al.* (1959) recommended that treated nematodes should be inoculated into nutrient agar, glucose peptone agar, Brewer's thioglycolate broth (for anaerobes) or Sabouraud's agar. One set is incubated at 37°C for 1 week and the other at room temperature for 14 days, although most bacterial or fungal contaminants are detected after 24 h. Ko *et al.* (1996) used nutrient glucose peptone, and PDA. Elhadi *et al.* (2018) used R2A (Merck) for bacterial growth and potato extract glucose agar for fungal growth.

6.5 Plant Tissue Culture

Axenic culture of sterile seedlings, excised roots or other plant tissue piece grown in artificial media has enabled monoxenic *in vitro* cultures of plant-parasitic nematodes to study different aspects of nematodes biology. Differences in pathogenicity or host preference and mutants have not been reported for plant nematodes propagated on callus for many generations, but development and reproduction could be affected as shown by the sex ratio changes of *D. dipsaci* (Viglierchio and Croll, 1968), and the rate of development of *M. incognita* when the concentration of medium constituents were varied (McClure and Viglierchio, 1966). Citrus leaf callus tissue culture (Inserra and O'Bannon, 1975), banana fruit callus (Brown and Vessey, 1985), carrot (Reise *et al.*, 1987) and excised soybean roots (Huettel, 1989) have been investigated for monoxenic culturing of *R. similis* (Elsen *et al.*, 2001). Modified Murashige and Skoog (1962) medium has enabled culture of *D. destructor* on groundnut callus tissue (van der Walt and De Waele, 1989) and *M. incognita* on peach plantlets (Huettel and Hammerschlag, 1986; Hashmi *et al.*, 1994). Species of root-knot nematodes (e.g. *M. incognita*, *M. javanica*) have been cultured on excised tomato roots grown on a medium based on Murashige and Skoog (1962), Skoog and Tsui (1948) and White (1963) media (Orion *et al.*, 1980), and hairy root cultures on Gamborg's B5 medium plus vitamins (Gamborg *et al.*, 1968; Verdejo *et al.*, 1988; Table 6.1). *Zygotylenchus guevarai* has been cultured on excised parsley roots (*Petroselinum crispum*) on Gamborg's B5 medium in 1.5% (Karakaş, 2007). The influence of nutrient salts, gelling agents, sucrose concentration and pH of the medium can affect the development of nematode-induced galls. Hutangura *et al.* (1998) optimized *in vitro* culture conditions of tomato seedlings to yield high *in vitro* rates of infection by *M. javanica*; they also pointed out that different species of *Meloidogyne* can react differently to the same nutritional conditions of the same plant, and the growth medium should be optimized for each species.

Mitkowski and Abawi (2002) examined monoxenic culturing of *M. hapla* on tomato root tips transformed with *Rhizobium rhizogenes* (see also Section 6.5.1) and non-transformed onion and dandelion root tips and found that, although nematode populations established on all systems, the onion root culture was the most effective method. The use of a pre-induction medium containing the hormone α-naphthaleneacetic acid was necessary for the production of onion root culture systems but not for the establishment of tomato or dandelion root cultures.

6.5.1 Roots of whole seedlings on agar

In general, seedlings obtain sufficient nutrient from their seeds to allow root growth on agar for several days or even weeks. Khera and Zuckerman (1963) placed concentrated extracts of *Aphelenchus decalineatus*, *Ecphyadophora tenuissima*, *Dorylaimus* spp., *Hemicycliophora similis*, *Helicotylenchus erythrinae*, *Tetylenchus joctus*, *Tylenchus agricola*, *T. bryophilus*, *Paratrichodorus minor*, *Tylencholaimus proximus* and *Tylenchorhynchus claytoni* from soil, or hand-picked and sterilized specimens, close to the roots of 3–7 day old seedlings of 13 different plants growing on 1% water agar in Petri dishes, inverted the dishes and

Table 6.1. Gamborg's B5 Basal Medium with minimal vitamins.

Distilled water	800 ml
1× Gamborg's B5 medium	3.2 g
Sucrose 2%	15 g
Bacto Agar	20 g

observed the feeding habits of nematodes. Russell and Perry (1966), Sutherland and Adams (1965) and Sutherland (1967) have also used this technique. Zuckerman (1961) placed a cover glass on the agar over the roots, inverted the Petri dish, and kept it in the dark so that roots grew down to the cover glass and could be examined under an oil-immersion objective; alternatively, a block of agar with seedling and nematodes can be placed in a deep-well slide with a cover slip for observation.

Monoxenic cultures of *P. penetrans* have been established on roots of white clover seedlings (Chen *et al.*, 1961) and strawberry seedlings (Chen and Rich, 1962) growing in tubes on agar containing modified Hoagland's nutrients (Hoagland and Arnon, 1950). Dropkin and Webb (1967) grew axenic tomato seedlings on agar slants of modified White's medium for studying resistance to *Meloidogyne* spp. Wyss (1970, 1971) observed the feeding of *P. penetrans*, *Rotylenchus* spp., *Tylenchorhynchus* spp., *Longidorus* spp. and trichodorids on strawberry seedlings on water agar and *Trichodorus* spp. feeding on rape and tobacco seedlings. The feeding of *Xiphinema* and *Longidorus* spp. on seedlings of grape (*Vitis vinifera*), annual nettle (*Urtica urens*) and tree-leaved bur marigold (*Bidens tripartita*) was studied by Cohn (1970), and the feeding of *P. minor* on tomato seedlings by Högger (1973). Müller (1978) used *Raphanus sativus* seedlings on agar to check for resistance to *Heterodera schachtii*.

The importance of the source of plant seedlings in studies of feeding site development should be considered, since excised roots differ from roots of monoxenically grown seedlings; they do not show a normal geotropic response, lose their ability to form secondary vascular tissue and their biochemical composition is altered. To overcome the latter issues, Hutangura *et al.* (1998) developed a protocol to optimize culture condition for *in vitro* infection of tomato seedlings with *M. javanica*. Hatching from *in vitro* egg masses produced by this method was much more synchronous than hatching from surface-sterilized eggs, most J2 emerging in the first 4 days. The protocol for tomato seedlings for *in vitro* culturing of *M. javanica* is described in the Section 6.5.1.1.

6.5.1.1 *Tomato seedlings for* in vitro *culturing of* Meloidogyne javanica

INOCULUM PREPARATION; SURFACE-STERILIZED EGG MASSES. Working in a laminar flow cabinet, 21 egg masses of *M. javanica* were surface sterilized in 600 µl of dH_2O in a polypropylene tube (1.5 ml), by adding 600 µl of 5% NaOCl (final concentration 2.5%) and shaking vigorously for 4 min. The egg suspension was centrifuged at 1000 *g* for 5 min and the supernatant removed with a micropipette. Then, 1% (w/v) $HgCl_2$ was added at a concentration of 0.2% and the suspension shaken gently for 4 min before being centrifuged at 1000 *g* for 5 min; the supernatant was removed and the eggs re-suspended in 200 µl of sterile dH_2O. The suspension was transferred into a 10 ml, capped conical centrifuge tube. The eggs were washed twice with 10 ml of sterile dH_2O for 5 min, followed by centrifugation. After re-suspension in 1 ml of sterile water, the eggs were ready for inoculation on *in vitro* cultured tomato plants, which allowed production of sterile egg masses and J2.

CULTIVATION OF TOMATO PLANTS. Tomato 'Grosse Lisse' seeds were surface sterilized by soaking in 70% ethanol for 15 min (25 ml per 100 seeds). After removing the ethanol, one volume of 2.5% NaOCl and a drop of Tween 20 were added, and seeds incubated for 20–30 min. The seeds were washed in a sterile sieve (1.2 mm mesh) with 500 ml of sterile dH_2O and placed in Petri dishes (9 cm diam.) containing *ca* 6 mm of solidified growth medium (quarter-strength Murashige and Skoog, 0.5% sucrose, pH 6.4, and 0.6% Phytagel®). Ten seeds were distributed randomly in each Petri dish, sealed with Parafilm and kept at 28°C with 16:8 h light:dark. Seven to 10 days after sowing, each Petri dish was inoculated with 400 eggs of *M. javanica* in 100–200 µl of sterile inoculum, plates were sealed and kept at 28°C with 16:8 h light:dark. Nematodes completed their life cycle 5–7 weeks after inoculation.

STERILE EGG MASS PRODUCTION FOR EGGS AND J2 INOCULA. Four sterile egg masses of *M. javanica* produced *in vitro* cultured tomato plants as described above were placed into one 60 µl droplet of sterile dH_2O and up to six droplets were placed in one Petri dish, sealed with Parafilm and incubated for 24–48 h at 28°C. Newly hatched J2 were harvested with a micropipette and inoculated onto fresh tomato seedlings without requiring further sterilization. After re-addition to egg masses of the removed volume of sterile water they were incubated for further hatching. Egg masses of a light brown colour gave the greatest hatch and harvesting

occurred approximately 5 weeks after infection of the culture. Older, dark brown egg masses (8 weeks after infection) showed reduced hatching rates.

6.5.1.2 Ditylenchus angustus *cultured on axenic rice seedlings*

Plowright and Akehurst (1992) surface-sterilized hulled rice seeds 'IR36' in $HgCl_2$ (0.1% w/v) for 30 min, rinsed them five times in sterile dH_2O, and then placed them on Gamborg's B5 basal medium (Gamborg *et al*., 1968; Table 6.1) supplemented with sucrose (2% w/v) and solidified with agar (1%) in 9 cm diam. plastic Petri dishes. The dishes were maintained at 23–27°C with a 12 h photoperiod and 30 days after sowing 20 *D. angustus* in 5 µl of sterile dH_2O were inoculated onto a leaf base adjacent to a new emergent leaf. Petri dishes were sealed immediately after inoculation and returned to the controlled environment.

6.5.1.3 Xiphinema index *cultured on tomato and fig seedlings*

Wyss (1978) studied the root and cell responses to feeding by *X. index* on seedlings of *Ficus carica* and *Solanum lycopersicum* 'Haubners Vollendung'. Seeds were soaked overnight in dH_2O, surface sterilized for 20 min in a filtered 4% $Ca(OCl)_2.4H_2O$ solution and washed for 1 h in sterile dH_2O and transferred onto 1% aqua destillata (dH_2O free from trace elements, salts and microorganisms) agar for germination in daylight (tomato) and darkness (fig) at 25–28°C. After the radicle emerged (3–4 weeks for fig and 2 days for tomato) the seeds were transferred onto 0.6% aqua destillata agar in Petri dishes and a few drops of Hoagland's solution No. 1 were added. Fine sand particles were scattered over the still liquid agar. Batches of 50 nematodes were surface sterilized by placing them for 90 min in a 0.03% sodium azide (NaN_3) solution contained in staining blocks. After the treatment, the NaN_3 solution was removed and replaced with two washes of sterile water. The nematodes were transferred with a sterile micro-needle to a 0.06% agar containing growing fig or tomato seedlings. About 60% of the treated nematodes survived and dispersed in the agar. Plates were sealed with Parafilm and kept at 25 ± 1°C at low light intensity of 700 lux (16 h day^{-1} exposure). Bleve-Zacheo and Zacheo (1983) made a similar study but fig seedlings were grown on 2% agar.

6.6 Explant Culture

Explants are small pieces of plant parts or tissues that are aseptically cut and used to initiate a culture in a nutrient medium. The term 'explant' can be applied to samples obtained from any part of the plant but, for culturing plant-parasitic nematodes, excised roots are the principal basis. To obtain excised root, sterilized seeds are germinated on a filter paper or on agar for 2–5 days; when the primary root is 2–5 cm long, the distal portion is excised 1 cm from the tip and placed in a nutrient solution, usually White's (1943a, b) or a modification thereof (Table 6.2), or in this solution plus 0.75–1.0% agar, where they will continue to grow and proliferate. Variations include placing the germinating seeds on nutrient agar and, when the primary root is 2–4 cm long, removing the seed leaving the radicle to grow. For more techniques and media details, see White (1943a, b, 1963), Street (1973) and Evans *et al.* (1984); many culture media are commercially available. Clean stock materials should be free of viruses and fungi.

Excised roots of various plants can be established in suitable plant tissue culture media under axenic conditions in Petri dishes or flasks (De Ley and Mundo-Ocampo, 2004). The explant material is surface sterilized via multiple NaOCl and alcohol washes, then washed in sterilized water and placed on a growth medium containing sucrose and one or more plant-growth regulators. Usually, the medium is thickened with agar to create a gel that supports the explant during growth. Some plants are easily grown on simple media, but others require more complicated media for successful growth. Although agar is the most common substrate, other gelling agents can be used instead, e.g. Gellan gum (Eyre and Caswell, 1991) and Pluronic F127 (Ko and Van Gundy, 1988; Ko *et al*., 1996). Sterile nematodes or eggs can be added to the excised roots in agar; if this is done in Petri dishes or other suitable containers their feeding habits and consequent effects can be observed.

Feder (1958) cultured *R. similis* on excised roots of *Hibiscus esculentus*. Bavaresco and Walker (1994) established *X. index* in aseptic dual culture and developed a quick method for surface sterilization that

Table 6.2. Modified Whites's agar medium (Hooper, 1986).

Na_2SO_4	800.0 mg
$Ca(NO_3)_2.4H_2O$	400.0 mg
$MgSO_4.7H_2O$	180.0 mg
KNO_3	80.0 mg
KCl	65.0 mg
$ZnSO_4.7H_2O$	6.0 mg
$MnSO_4.4H_2O$	4.5 mg
H_3BO_3	0.375 mg
KI	3.0 mg
Ferric tartrate	40.0 mg
Glycine	3.0 mg
Thiamine hydrochloride	0.1 mg
Ca panthotenate	2.5 mg
α-naphthaleneacetic acid[a]	0.1 mg
2,4-Dichlorophenoxyacetic acid	2.0 mg
Sucrose	20.0 g
Agar	15.0 g
Coconut milk[a]	150.0 ml
Distilled water	to 1000.0 ml

[a]Omitted by Krusberg and Blickenstaff (1964).

allowed screening grapevines for *X. index* resistance on *Vitis rupestris* 'St. George' roots. The culture media that allowed survival, feeding and reproduction of *X. index* consisted of Nitsch and Nitsch (1969) salts and vitamins medium with 15 g l^{-1} sucrose and 6 g l^{-1} agar; the inoculated excised roots were cultured in the dark. Winterhagen *et al.* (2007) developed an *in vitro* dual system for grapevines and *X. index* as a tool to investigate GFLV grapevine fanleaf virus infection. Grapevine cuttings were cultivated in autoclaved sand substrate supplemented with half-strength Murashige and Skoog (1962) medium (pH 5.8); *X. index* was inoculated 3 weeks after culture initiation. Nematodes were washed and rinsed several times with dH_2O but no additional disinfection treatment was given before inoculation, as described by Bavaresco and Walker (1994), because of reported negative effects on *X. survival* and feeding behaviour.

Mesocriconema xenoplax was grown in monoxenic cultures on excised roots of crimson clover (*Trifolium incarnatum*), carnation (*Dianthus caryophyllus*), western sand cherry (*Prunus besseyi*) and tomato grown on Gamborg's B5 medium (Wescott and Hussey, 1992). Crimson clover was the most suitable explant host and nematodes remained active in crimson clover cultures 15–20 weeks after establishment but roots had stopped growing by *ca* week 12. Some specific examples of explant cultures are given below.

6.6.1 Tomato root explants

Early work culturing *Meloidogyne* on excised tomato roots included Tyler (1933) and Peacock (1959) and the analysis of the *M. incognita* host–parasite relation by single juvenile inoculations (Dropkin and Boone, 1966). Also, on tomato, Widdowson *et al.* (1958) obtained mature females of *G. rostochiensis* and Prasad and Webster (1967) studied the effect of temperature on the rate of development of *N. aberrans s. l.* Ko *et al.* (1996) cultured endomigratory plant-parasitic nematodes on root explants of tomato 'Rutgers' on Murashige and Skoog (1962) medium supplemented with 2% sucrose prepared in either 1.5% (w/v) agar or 0.25% Gelrite; plates can be stored at 4°C until use. Two tomato seeds, surface sterilized (Ko and Van Gundy, 1988), were placed near the edge of the Petri plates containing the medium and allowed to germinate in the dark at 25°C for 3–4 days and radicles were 2–3 cm long. The epicotyls were removed with a sterile scalpel and roots were allowed to grow for another 3–5 days. Postma *et al.* (2012) produced *in vitro* tomato or potato explants on B5 medium (3.29 g l^{-1} Gamborg B5, 20 g l^{-1} sucrose, 15 g l^{-1} bacto agar; pH 6.2) at 24°C and 16 h:8 h day:night photoperiod for 3 weeks prior to inoculation with *G. rostochiensis* J2.

6.6.2 Maize root explants

Maize root explants can be maintained in Petri plates containing Murashige and Skoog medium using 1.5% (w/v) agar or 20% (w/v) polyol (Pluronic F127) as a support base for short-term culturing of *Pratylenchus* spp. Inoculation of the nematode (*ca* 250 mixed life stages) is made when the explants are 5 days old (Ko *et al.*, 1996). *Pratylenchus* stock cultures and preparation of inocula can be maintained according to Huettel (1990).

Mountain (1955) cultured *P. neglectus* on excised maize, tobacco and red clover roots and Tiner (1960) cultured *P. penetrans* on excised maize roots on agar and described an apparatus (Tiner 1961, 1966) in which, once monoxenic root cultures have been established, they can be maintained and nematodes collected over a period of several weeks; the apparatus used to be available commercially as the Melpar-Tiner storage trap. Excised maize roots have also been used to culture *Belonolaimus longicaudatus* (Huang and Becker, 1997, 1999).

6.6.3 Cucumber excised roots

A long-term method to maintain and increase *M. arenaria*, *M. javanica* and *M. incognita* on cucumber excised roots was described by Díaz-Manzano *et al.* (2016). Fifty seeds of *Cucumis sativus* 'Hoffmanns Giganta' are surface sterilized with 40 ml of NaOCl for 45 min and then washed five times with sterile dH_2O in a laminar flow cabinet. Five seeds in a Petri dish (14 mm diam.) are sown in solid modified Gamborg's B5 medium supplemented with 3% sucrose; each plate is double sealed with Parafilm and Micropore® before wrapping with aluminium foil. Plates are kept at 4°C for 2 days, and then in darkness at 26°C for 21 days in a growth chamber. The etiolated aerial parts of the seedlings are removed before inoculation with the J2. Four days before inoculation, 50 sterile *Meloidogyne* egg masses (amber in colour) are picked up from 2-month-old cucumber *in vitro* root cultures. Egg masses are placed in a sterile cell strainer with a 70 µm nylon mesh inside a beaker filled with 5 ml of sterile tap water. Four days later, 1 ml suspension of freshly hatched J2 is used to inoculate fresh cucumber plates every 23 days. One Petri dish provides 50 egg masses to inoculate ten new cucumber plates. The sterile J2 produced by this method have, for example, been used successfully in *in vitro* experiments to infect different genotypes of *Arabidopsis* (Cabrera *et al.*, 2014, 2015, 2016).

6.6.4 Cyst nematodes on excised roots

Heterodera glycines has been cultured on excised soybean, *Glycine max*, roots (Lauritis *et al.*, 1982; Eisenback and Zunke, 1998). Seeds are soaked in a 1–2% NaOCl solution for 10 min, rinsed in sterile dH_2O and placed on 1.5% water agar in Petri plates. After germination, the 2–4 cm root tip radicles are aseptically excised and transferred to a suitable growth medium where lateral roots develop and can be inoculated with sterile eggs or J2.

Heterodera schachtii has been cultured on excised roots of sugar beet, *Beta vulgaris* (Moriarty, 1964; Johnson and Viglierchio, 1969) and oil-seed rape, *Brassica napus* (Müller, 1978). De Ley and Mundo-Ocampo (2004) cultured *H. schachtii* using a modified protocol from Cordero and Baldwin (1990). Seeds, surface sterilized in 10% commercial bleach for 10 min, are rinsed with sterile water and plated on 1% agar. After germination in the dark, seedlings are transferred to a Petri dish containing Gamborg's B5 medium plus White's organics (pH 6.5) with 0.25% Gelrite. J2 are disinfected by rinsing them four rinses in dH_2O and 10 min incubation in saturated aqueous Rifampicin® antibiotic solution; prior to inoculation, J2 are rinsed in sterile water. Young roots are inoculated with *ca* 25 surface-sterilized J2; plates with infected roots are incubated at 25°C in a growth chamber (16 h; 300 lux cycle). De Ley and Mundo-Ocampo (2004) modified the protocol of Cordero *et al.* (1991) to culture *Cactodera cacti* on Christmas cactus (*Schlumbergera* spp.) roots.

6.6.5 Leaf cultures

Sanwal (1959) set single leaves of chrysanthemum in wet sand in a moist, ventilated atmosphere. One or more *Aphelenchoides ritzemabosi* were placed on each leaf in minute droplets of water that were then allowed to dry. At intervals, the leaves were sprayed with water from an atomizer to maintain high humidity.

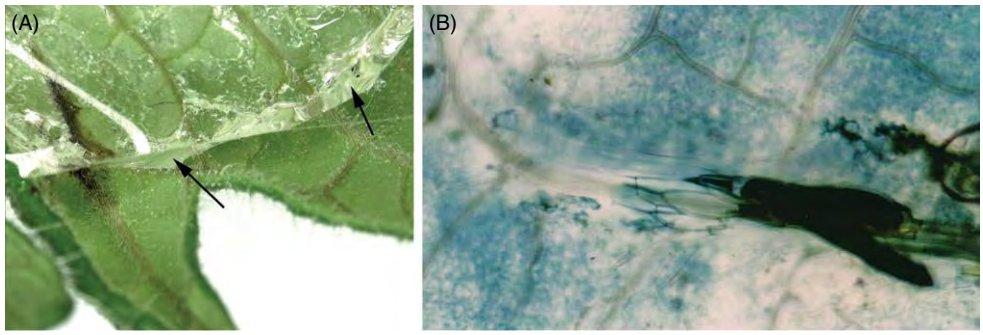

Fig. 6.2. Tomato leaf inoculated with *Globodera rostochiensis*. **(A)** Second-stage juveniles were initially deposited in a drop of water on the abaxial surface of the leaf next to the rachis and leaf veins and a strip of Biofolie 25 (Teflon-type gas permeable membrane) placed on top of the drop (arrows). (Photo: copyright Rothamsted Research Ltd.) **(B)** Third-stage juvenile. Leaves and nematodes were stained with trypan blue lactophenol 7-14 days after inoculation. (Photo: Rosa H. Manzanilla-López.)

Symptoms appeared on the leaves after 7–11 days and nematode populations increased. Cabrera Poch *et al.* (2006) modified this technique to carry out *G. rostochiensis* J2 foliar inoculation on tomato to induce a hypersensitive reaction on the plant tissue. The J2 (600 J2 leaf^{-1}) were deposited in a drop of water on the abaxial surface of the leaf, next to the rachis and leaf veins, and a strip of Biofolie 25 (Heraeus, Germany), a Teflon-type gas permeable membrane, was placed on top of the drop (Fig. 6.2A). The inoculated leaves were kept in a Petri dish in a controlled temperature room for 7–14 days at 23°C (not directly exposed to light), taking care that both the tissue enveloping the tip of the rachis of the leaf and the filter paper in the Petri dish were always wet. Eight days later, the leaf from the Petri dish was removed and the Biofolie 25 strip peeled off from the leaf surface (Fig. 6.2B).

Shepperson and Jordan (1968) established *M. incognita* on aerial parts of begonia and tomato plants by inoculating midribs of leaves with sterile eggs or juveniles using glass capillary tubes.

6.6.6 Potato tuber, potato plug and storage root cultures

Williams (1963) surface-sterilized small potato tubers, removed eye tissue to prevent sprouting, and cut the tubers in half. A concentrated suspension of *G. rostochiensis* J2 was added to the cut surface and the pieces were buried in washed sand kept moist by the addition of 20 ppm $CuSO_4$ solution. Females developed on the cut surface and could be counted after about 6 weeks. Steele (1972) cultured *H. schachtii* on slices of storage roots of sugar beet, red table beet, radish, turnip and rutabaga. Eggs and juveniles were added to the slices, which were then covered with moist granulated agar or soil; adults developed in 12–62 days at 24°C.

Seinhorst and Bels (1951) made holes about 5 mm deep by 2–3 mm diam. in the surface of clean tubers in which they placed a small quantity of *D. destructor* suspension. The cavity was sealed with heated wax, which set immediately, and the heat was insufficient to damage either nematodes or potato tissue. The tubers were stored in wide-mouthed jars with damp filter paper to maintain a moist atmosphere.

Dallimore (1966) embedded aseptic plugs of potato in potato dextrose, cornmeal or water agar and inoculated them with a piece of tissue from a young lesion of a potato infected with *D. destructor*. After 5 weeks, the plug was infected and pieces could be used to inoculate further plugs or potato plants. Pupavkina (1971) used slices of potato or carrot for culture of *D. destructor*.

6.7 Callus Tissue Culture

A callus is a mass of undifferentiated parenchymatous cells that is formed when a living plant tissue is placed in an artificial growing medium under favourable conditions. Callus is a useful method for culturing large numbers of stylet-bearing nematodes, although *Meloidogyne* and *Heterodera* species do not reproduce well

on completely undifferentiated callus tissue (Zuckerman, 1971; Paul *et al*., 1987). The growth of callus varies according to growth regulators auxin and cytokinin supplied in the culture medium. The callus growth consists of three different stages: (i) the rapid production of callus after placing the explants in culture medium; (ii) induction of adventitious organs when the callus is transferred to medium containing growth regulators; and (iii) gradual exposure of the new plantlet to the environmental conditions. The undifferentiated cells of callus tissue provide a ready food source, and callus is often a better host than the differentiated tissue from which it is derived (Schroeder and Jenkins, 1964); tissues from some normally resistant plants also support nematode reproduction when in callus form (Webster, 1966; Webster and Lowe, 1966).

The callus is usually grown on an agar medium, commonly that based on White's (1943a,b) medium modified by Hildebrandt *et al.* (1946) and Krusberg (1961). The following formulation, recipe (Table 6.2) and preparation method is from Hooper (1986). Stock solutions are prepared in glass dH_2O (more dH_2O should be passed through an ion-exchange column) and stored separately to prevent interactions. About 700 ml of dH_2O is boiled and the agar dissolved. The remaining solutions are added and the total made up to 1 l. The medium is placed in 2.5 × 15 cm Pyrex tubes (15 ml $tube^{-1}$), which are capped with aluminium foil and autoclaved at 1.05 kg cm^{-2} for 15 min. The tubes are allowed to cool on the slant. Flasks or bottles may be used instead of tubes.

Axenic tissues and axenized nematodes must always be used to avoid contamination of callus tissue or any other tissue or plant cultured *in vitro*. Sterile seeds are usually germinated on water agar, or on sterile moist filter paper, and when growth is 2–3 cm long, two to six seedlings are transferred to the nutrient agar in a tube or Petri dish. The election of a seed sterilization protocol would largely depend on the seed as they may require scarification by acid treatment as for *Lotus japonicus* (Lohar and Bird, 2003; Cabrera Poch *et al*., 2007). Elhady *et al.* (2018) surface-sterilized soybean and tomato seeds with 1.5% NaOCl for 15 min and rinsed five times with sterile deioinized water; seeds were germinated afterwards on paper tissue under sterile conditions for 5 days.

The tissues are usually allowed to grow for 2–3 weeks at 20–25°C before nematodes are added. The tubes are kept capped with aluminium foil, which is sealed with tape to prevent evaporation, and cultures will often last for several months. Subcultures can be made by adding a small piece of nematode-infested callus to fresh callus. Nematodes can be extracted by breaking up the callus in water and placing it on a filter. To avoid contamination, nematodes for cultures are often extracted and stored in sterile dH_2O, and the pH of water is often low.

Krusberg (1961) used a modified White's medium for growing lucerne (alfalfa) callus on which he cultured *D. dipsaci*, *Aphelenchoides ritzemabosi*, *P. penetrans*, *P. zeae* and, less successfully, *Tylenchorhynchus capitatus* and *Hoplolaimus galeatus*. Schroeder and Jenkins (1964) found that the above medium, without NAA (α-naphthaleneacetic acid) and coconut milk, was better for reproduction of *D. dipsaci*, *P. penetrans* and *P. zeae* on lucerne callus; for *D. dipsaci*, 0.5 ppm kinetin was added. Webster and Lowe (1966) supplemented the medium with $CuCl_2.2H_2O$, 2 mg l^{-1}, $Na_2MO_2.2H_2O$, 2.5 mg l^{-1} and $CaCl_2$, 220 mg l^{-1} for growing lucerne, red clover and potato callus, but used 6 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) for rose, apple and pea callus; they also found that reproduction of *A. ritzemabosi* and growth of lucerne callus was greater on a medium with 0.125 mg l^{-1} of 2,4-D. Webster (1966) cultured oat callus on a different medium and the cultures grew better in the light than in the dark. Riedel and Foster (1970) considered that Krusberg's medium with coconut milk was somewhat better than modified White's medium (+0.1 mg NAA and 2 mg 2,4-D l^{-1}) for *D. dipsaci* on onion callus. Faulkner *et al.* (1974) preferred a modified White's medium plus coconut milk for the mass culture of *D. dipsaci* on lucerne callus. Viglierchio *et al.* (1973) gave detailed observations on callus culture and faba bean stem culture and concluded that Henk's medium, which they described, was more suitable for a wide variety of callus tissues than the above-mentioned media. Reidel *et al.* (1973) used a simplified medium (20 g sucrose, 5 g yeast extract, 2 mg 2,4-D and 10 g Difco-Bacto agar in 1 l dH_2O), which allowed reproduction of *D. dipsaci* and *P. penetrans* on onion and lucerne callus, respectively; this medium was also better than some others for reproduction of *Bursaphelenchus xylophilus* on lucerne callus (Tamura and Mamiya, 1976). Zakrzewski and Zakrewska (1976) concluded that Krusberg's medium with 2,4-D and corn (*Zea mays*) milk, instead of coconut milk, was best for the culture of *D. dipsaci* on red clover callus. Bingefors and Bingefors (1976) and Eriksson (1980) gave details for rearing *D. dipsaci* inoculum for plant breeding purposes. Other successful cultures include *D. destructor* on potato, carrot, clover, tobacco and groundnut callus (Darling *et al*., 1957; Faulkner and Darling, 1961; van der Walt and De Waele, 1989); *A. ritzemabosi* on callus of eight plants of which tobacco was the best (Dolliver *et al*., 1962);

Tylenchus agricola and *Tylenchorhynchus claytoni* on lucerne callus (Khera and Zuckerman, 1963); *Aphelenchus avenae* on tobacco (Barker, 1963) and on carrot, periwinkle and tomato (Barker and Darling, 1965); *R. similis* on grapefruit, okra and lucerne callus (Myers *et al.*, 1965); *Dolichodorus heterocephalus* on corn-root callus (Paracer and Zuckerman, 1967); *Telotylenchus indicus* on lucerne callus (Khera *et al.*, 1969); and *Paratylenchus projectus* on clover callus (Townshend, 1974). Inserra and O'Bannon (1975) reared *R. similis* and *P. coffeae* on citrus leaf callus and citrus roots.

6.7.1 Alfalfa callus

Alfalfa (*Medicago sativa*) is considered a versatile plant for callus culture and an efficient means for maintenance and propagation of migratory nematodes. Alfalfa can be cultured on modified White's medium in 1.5% agar or 0.25% Gelrite (Ko *et al.*, 1996). Nematodes cultured on alfalfa callus include *D. dipsaci* (Krusber, 1961; Hajihassani *et al.*, 2017) and *Aphelenchoides ritzemabosi* (Krusberg, 1961). Roman and Hirschmann (1969) propagated aseptically six species of *Pratylenchus* on alfalfa callus following the method of Krusberg but using 2,4-D as growth regulator, and Riedel *et al.* (1973) used a more simplified nutrient agar than the Krusberg's medium to culture *P. penetrans*. The latter species can multiply faster on callus than on excised tomato roots (Schroeder and Jenkins, 1964). *Radopholus similis* has also been cultured successfully on alfalfa callus (Myers *et al.*, 1965; Castro and Ferraz, 1990; Ko *et al.*, 1996). According to Elsen *et al.* (2001), alfalfa callus cultured on White's medium was a good host for *R. similis* but for *P. penetrans* the modified White's medium (containing 0.2 ppm NAA and 2 ppm 2,4-D) proved to be as good as Krusberg's medium (Riedel and Foster, 1970).

Elsen *et al.* (2001) surface-sterilized alfalfa seeds with a 15 min soak in concentrated H_2SO_4, rinsed four times with sterile dH_2O, followed by another 15 min soak in $HgCl_2$ (1000 ppm in 30% ethanol) and four rinses with sterile dH_2O. Sterile seedlings were produced on agar (10 g sucrose, 2 g yeast agar, 1000 ml water) plates. Four-day-old alfalfa seedlings were placed on 14 ml aliquots slants prepared from White's medium modified by adding 0.2 ppm NAA and 2 ppm 2,4-D. Seven to 10 days later, the calli were transferred to Petri dishes containing the same medium. The nematode culture on alfalfa callus was initiated with *R. similis*, previously cultured on carrot discs, and surface sterilized for 2 min in 0.01% $HgCl_2$, followed by two rinses of sterile dH_2O. Twenty females were inoculated on each alfalfa callus with a sterile micropipette; Petri dishes were incubated at 28 ± 0.5°C in the dark and 5 weeks later the nematodes began moving from the callus. To maintain sterile *R. similis* stock cultures, fresh alfalfa calli were infected with a small piece of *R. similis* infected callus. To extract the nematodes, the callus was chopped and put on a sterile 70 µm pore sieve placed on a sterile watch glass containing sterilized water. Within 48 h the nematodes migrated through the sieve into the water and collected from the bottom of the watch glass. Nematodes were placed on PDA and NA (nutrient agar) to test for bacterial and fungal contamination.

Riedel *et al.* (1973) used a simplified NA for alfalfa and onion callus tissue culture. One-week-old sterile onion and alfalfa seedlings were cultured for 2 weeks in a nutrient medium (20 g sucrose, 5 g yeast extract, 2 mg 2,4-D, 10 g Difco-Bacto agar, 1000 ml dH_2O) in 25 × 150 mm tubes. Onion and alfalfa callus tissues were inoculated with *D. dipsaci* and *P. penetrans*, respectively, and cultures were maintained in the dark at 23°C. Nematodes were extracted at 8 (*D. dipsaci*) and 10 (*P. penetrans*) weeks from cultures, producing populations of 10,500 nematodes $tube^{-1}$ for *D. dipsaci* and 20,460 nematodes $tube^{-1}$ for *P. penetrans*, comparable to those expected with Krusberg's medium (Riedel *et al.*, 1973).

6.7.2 Banana fruit callus

Brown and Vessey (1985) used banana fingers (*Musa acuminata* AAA 'Grande Naine') 8 weeks after shooting to produce the callus on Murashige and Skoog's (1962) modified medium (5 g l^{-1} agar, 30 g l^{-1} sucrose, 1.0 mg l^{-1} indole-3-acetic acid, 0.5 mg l^{-1} benzyl adenine, 2 mg l^{-1} glycine, 0.4 mg l^{-1} thiamine HCl, 0.5 mg l^{-1} nicotinic acid and 0.5 mg l^{-1} pyridoxine HCl), pH 5.6. Ten ml aliquots were added to 25 × 90 mm glass tubes, covered with aluminium foil and autoclaved at 103 KPa (= 15 p.s.i) for 15 min. Banana fingers were surface sterilized in 0.5% NaOCl and, under aseptic conditions, pulp tissue was cut into cylinders (*ca* 10 mm in diam. and 4 mm thick) and placed on the surface of the culture medium. Tubes containing pulp tissue were

incubated at room temperature (24–29°C) in indirect sunlight for 14 days before inoculation with aseptic nematodes. Individual tubes containing *ca* 1 g of callus were inoculated each with 50 surface disinfected *R. similis* in aqueous streptomycin sulfate (10 g l^{-1}) and incubated at room temperature for 30 days. Tubes contained an average of 391 ± 21 *R. similis*.

6.7.3 Rice and wheat callus

To culture *D. angustus*, Plowright and Akehurst (1992) prepared callus tissues from *Oryza sativa* 'NC492' and 'IR36' (mature embryos) and 'Speaker' (immature inflorescences), *Triticum aestivum* 'Copain' and *T. monococcum* (immature embryos). Immature embryo tissues were placed on Murashige and Skoog's (1962) medium, pH 5.8, supplemented with sucrose (3% w/v) and 2,4-D acetic acid (0.1 mg l^{-1}) and solidified with agar (0.6% w/v). The callus tissues were maintained at 25°C and sub-cultured every 8–12 weeks. Gamborg's B5 supplemented medium supported better growth of rice mature embryo callus than Murashige and Skoog's medium.

6.7.4 Groundnut callus

Van der Walt and De Waele (1989) surface-sterilized leaves of 4-week-old *Arachis hypogaea* 'Sellie' for 30 s in 70% ethanol (containing Tween 20) and then for 15 min in 0.05% NaOCl. Leaves were rinsed four times in sterile dH_2O. In the laminar flow cabinet, 1 cm^2 leaf sections were transferred to 9 cm diam. Petri dishes containing 25 ml of a modified Murashige and Skoog (1962) medium (pH 5.7 ± 0.1), and autoclaved at 121°C and 108 KPa (= 1.05 kg cm^{-2}) for 15 min (Table 6.3). The resulting callus tissue cultures were incubated in the dark at 25°C for 4 weeks and, once their volume had increased, they were transferred to fresh Petri dishes and inoculated with aseptic nematodes.

Table 6.3. Modified Murashige and Skoog medium (Van der Walt and de Waele, 1989).

Substance	mM	mg l^{-1} [a]
NH_4NO_3	20.6	1650
KNO_3	18.8	1900
$CaCl_2.2H_2O$	3.0	440
$MgSO_4.7H_2O$	1.5	370
KH_2PO_4	1.25	170
KI	0.5	0.83
H_3BO_3	100	6.3
$MnSO_4.4H_2O$	100	22.3
$ZnSO_4.7H_2O$	30	8.6
$Na_2MoO_4.2H_2O$	1	0.25
$CuSO_4.5H_2O$	0.1	0.025
$CoSO_4.6H_2O$	0.1	0.025
Na_2EDTA	100	37.3
$FeSO_4.7H_2O$	100	27.8
Vitamins		
Inositol		100
Nicotinic acid		1
Pyridoxine.HCl		1
Thiamine.HCl		10
Sucrose		30 g l^{-1}
Casein hydrolysate		0.5 g l^{-1}
2,4 Dichlorophenoxyacetic acid		1
Agar		8 g l^{-1}
Distilled H_2O		Up to 1 l

[a]Note: sucrose, casein hydrolysate and agar are given as g l^{-1}.

6.7.5 Carrot callus

Reise *et al.* (1987) used callus tissue from 4-week-old surface-sterilized carrot discs for culturing *Pratylenchus* spp. Excised calli were transferred to Petri plates containing Gamborg's B5 medium plus 0.1 mg l^{-1} 2,4-D, 12.5 mg l^{-1} gentamycin sulfate, and 1.5% agar noble. The callus cultures were incubated at 28°C for 6–8 weeks and calli transferred to fresh plates. *Pratylenchus agilis*, *P. scribneri* and *P. brachyurus* were extracted for 18 h from maize root explants cultures and surface sterilized (Huettel and Rebois, 1985). Culture plates, containing *ca* 1.5 g callus growth, were inoculated with *ca* 100 nematodes of mixed life stages and incubated at 28°C for 60 days. Nematodes were extracted by transferring each callus to a flask containing 30 ml water and agitating for 1 h at 300 rpm. After agitation, water containing the callus and nematodes was collected on a 25.4 µm pore screen and transferred to a 50 ml beaker. Cultures remained viable for up to 5 months.

Carrot callus has also been used by Kaplan and Davis (1990). They dipped carrots in 95% ethanol and flamed them, then removed the outer tissues with a sterile scalpel. Each carrot was cut into 8 mm thick discs. Four discs (*ca* 6.5 g fresh weight) were placed in culture tubes (15.0 × 2.5 cm) and closed with plastic caps. When initial signs of callus formation were apparent (*ca* 3–4 weeks) carrot discs were inoculated with nematodes and maintained in an incubator at 26°C.

6.8 Carrot Disc Culture

There are several protocols for culturing plant-parasitic nematodes on carrot discs; it is the most commonly used *in vitro* technique for culturing *R. similis* (O'Bannon and Taylor, 1968; Huettel, 1985, 1990), although the nematodes can be contaminated easily (Elsen *et al.*, 2001). Carrot discs culture has also been used for mass culturing *D. dipsaci* (Viglierchio, 1971; Behmand *et al.*, 2017), *P. brachyurus* (O'Bannon and Taylor, 1968) and *P. vulnus* (Lownsbery *et al.*, 1967; Moody *et al.*, 1973). O'Bannon and Taylor (1968) cut discs 2–4 mm thick from carrots that were previously washed thoroughly, dipped in 95% ethanol and flamed to sterilize them. The discs were placed on 1% water agar and axenic *P. brachyurus* or *R similis* were pipetted on to the agar beside them or were added to the agar and the discs placed on top. Moody *et al.* (1973) cultured *P. vulnus* on carrot discs and recommended the use of freshly harvested carrots with tops to avoid bacteria and fungus soft rot; tops are then removed and the carrots scrubbed. Working in a laminar flow cabinet, they pared off the external tissue in a spiral pattern, flaming the knife before each contact with the carrot. As a carrot is pared, discs 10–15 mm in thickness are cut with the knife into sterile Petri dishes before transfer with forceps into culture jars. This procedure eliminates the need for water agar. Verdejo-Lucas and Pinochet (1992) found that nematode numbers recovered from carrot disc cultures varied greatly among five species of migratory nematodes (*P. neglectus*, *P. thornei*, *P. vulnus*, *R. similis* and *Zygotylenchus guevarai*) after 90 days of incubation in the dark at 26 ± 1°C.

Hajihassani *et al.* (2017) used fresh grocery carrots, washed them thoroughly with tap water and surface sterilized in a 6% (v/v) NaOCl solution for 2 min, then peeled and soaked them in 95% ethanol for 15 min. Then the carrot outer surface was thoroughly flamed, peeled and sliced into 4–5 mm thick discs. Kaplan and Davis (1990) found that culture longevity and contamination rates did not differ between cultures from carrots directly harvested from the field and those from carrots bought in grocery shops; carrots that are badly bruised or cut should be avoided. These authors extracted viable nematodes and eggs of *Radopholus* spp. by macerating infested carrot tissue with a mixture of 0.50% driselase and 0.50% cellulysin, w/v each, with 2.5 ml of enzyme solution g tissue^{-1}. Maceration slurries containing carrot tissue and nematodes were maintained in open flasks on a rotary shaker (175 rpm) at 26°C for 24 h. Nematodes and eggs were extracted from resultant culture slurries by flotation with $MgSO_4.7H_2O$ (S.G. 1.1).

A useful general protocol for carrot disc culture (Fig. 6.3) has been provided by Liza Alejandra González Jiménez, pers. comm. (Earth University, Guácimo, Limón, Costa Rica):

- Wash fresh carrots with running water, disinfected with detergent (Sterilex®) and leave to drain until dry.
- In a laminar flow cabinet and on top of a sterile paper towel, hold carrots with sterile forceps and sprinkle with alcohol and flame three times.

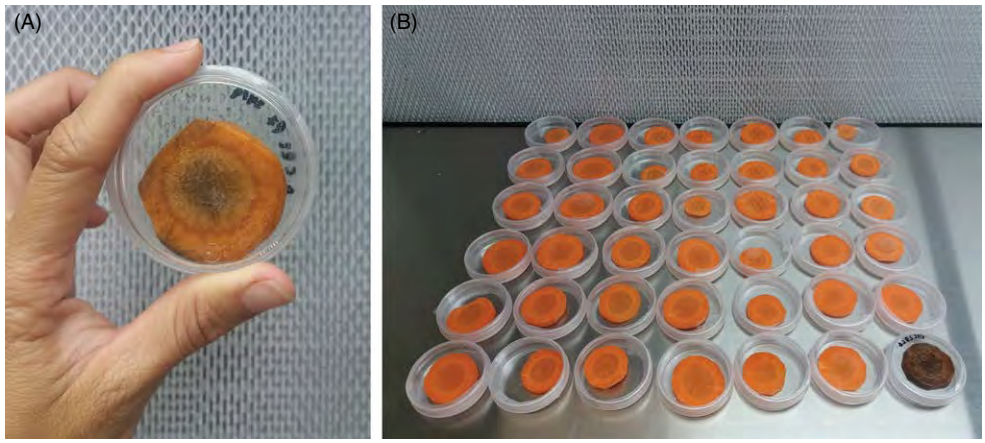

Fig. 6.3. Carrot disc culture. **(A)** *Radopholus similis* carrot disc culture. **(B)** Fresh carrot discs inoculated with *R. similis*. (Images courtesy of Alejandra González Jiménez, Earth University, Guácimo, Limón, Costa Rica.)

- On top of a new paper towel, peel each carrot from top to bottom with a previously flamed scalpel. Ensure the carrot is always held with the sterile tweezers and the scalpel is flamed every time the carrot skin is peeled; repeat the procedure until the carrot is 0.3 cm thick.
- Then, on a new paper towel, cut the carrot discs (*ca* 3–5 cm thick) and place discs into Petri dishes (60 × 15 mm), sealed with Parafilm and store at 4 to 8°C until use.

When the nematodes are visible on the inner surface of the Petri dishes and the perimeter of the carrot discs, they can be sub-cultured. Nematodes are removed in a laminar flow cabinet with sterile dH_2O in a pipette, passed through a small sieve (20 µm mesh), washed three times with 1 ml of sterile dH_2O each time, and then placed into a Syracuse watch glass containing 3 ml of 0.01% $HgCl_2$, and left for 2 min. After washing three times with sterile dH_2O, the nematodes are placed in a test tube with 3 ml of sterile water to which is added 3 ml of 0.06% streptomycin solution, using a sterile syringe and bacterial filter, and left for 3 h. After washing three times with 1 ml of sterilized water the mixture of nematodes and water (3–5 ml) is obtained, and fresh carrot discs can each be inoculated with 3 to 4 drops of the nematode suspension.

6.9 *In Vitro* Banana Plantlets

In vitro banana plantlets have been used to test *R. similis* pathogenicity to banana under controlled conditions (growth chamber) using nematodes monoxenically reared on carrot discs and then inoculated onto banana plantlets produced through *in vitro* micropropagation (Sarah *et al.*, 1993). Marin *et al.* (2000) described a standard assay method for screening for resistance of bananas to *R. similis* under glasshouse conditions. Banana plants from tissue culture, grown in 0.4 l Styrofoam cups containing sterilized sand as substrate, were maintained in the glasshouse for 4 weeks before inoculation. Two hundred *R. similis*, reared in monoxenic carrot disc culture, were used as inoculum for each container. Plants were kept in the glasshouse for an additional 8 weeks at about 27°C and 80% relative humidity after inoculation. Micropropagated banana plantlets are nowadays commercially available (Fig. 6.4) and selected accessions can be obtained upon request from The International Transit Centre (ITC, Katholieke Universiteit Leuven, Belgium), which hosts the International *Musa* Germplasm Collection.

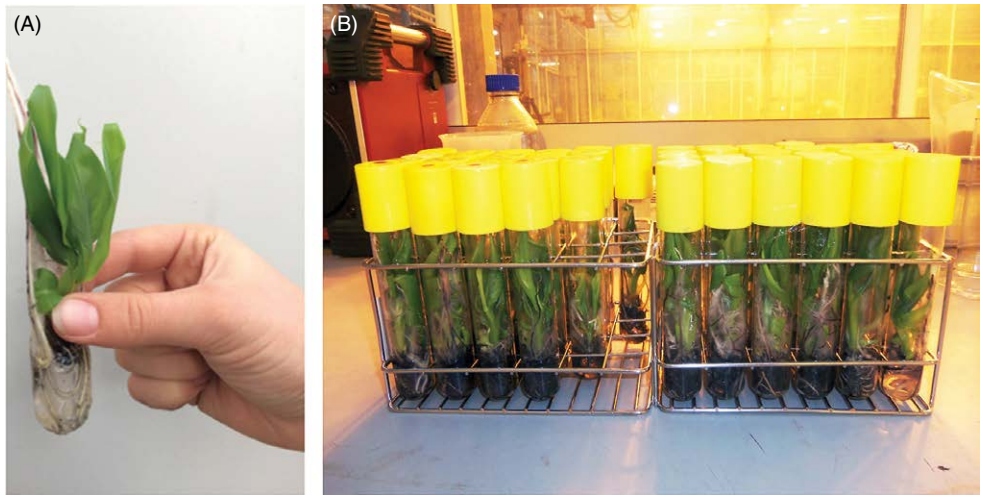

Fig. 6.4. *In vitro* banana plantlets. **(A)** Single rooted banana plantlet obtained from *in vitro* propagation. **(B)** Tubes with *in vitro* plants of 'Valery' (ITC 0048, Cavendish subgroup) supplied by the International *Musa* Transit Centre (ITC, https://www.bioversityinternational.org/banana-genebank/). (Images courtesy of Jassmine Zorrilla, Laboratory of Tropical Crop Improvement, Katholieke Universiteit Leuven, Belgium.)

6.10 Gnotobiotic Culture of Whole Plants

Methods for sterile culturing of higher plants can consist of one small container where the entire plant is enclosed or the roots maintained under sterile conditions while the shoots are allowed to grow freely (Zuckerman, 1971). The small, transparent, enclosed container allows nematodes to be placed in contact with a seedling grown in a tube (Fig. 6.5A,B) or a plate containing agar and make direct observation of feeding, penetration and symptom development, but such a system has relatively short duration due to nutrition needs of the plant and seedling overgrowing the container (see Manzanilla-López *et al*., 2011). Agar can be substituted by other (Fig. 6.5C) more natural substrates such as peat moss, sand or a mixture of loam, sand and active charcoal; however, the advantage of direct observations is lost (Zuckerman, 1971). Den Ouden (1960) described a sterile root apparatus involved growing plants with sterile roots in thin agar layers held in polythene bags and injecting sterile nematodes at the proper time. Klinkenberg (1963) used soil-less systems made of polyethylene bags containing thin agar layers for culturing *Rotylenchus uniformis*, *Pratylenchus crenatus*, *P. penetrans*, *Tylenchorhynchus dubius* and *H. similis*. DuCharme and Hanks (1961) cultured *R. similis* in large glass tubes on citrus seedlings growing in sterile sand and moistened with nutrient solution; the tubes were continuously aerated with moist sterile air. Zuckerman and Brzeski (1966) described methods for the study of plant-parasitic nematodes in gnotobiotic root culture in which plants were grown for some 3 months. Deubert *et al.* (1967) used a modification of Zuckerman and Brzeski's apparatus, having an enclosed irrigation chamber containing sterile nutrient solution. Feldmesser (1967) devised an *in vitro* method where the roots are implanted in agar while the seedling stem passes through a cotton-plugged hole cut in the top of a plastic Petri dish. Thus, the upper portions of the plant are exposed to the air, while the roots are held under sterile conditions. Hahn (1967) described a sterile-culture chamber for plants, and Polychronopoulus and Lownsbery (1968) established monoxenic cultures of *H. schachtii* on sugar beet seedlings in a plant-growth medium in narrow-mouthed Mason® jars. Rössner (1971) used hermetically sealed glass jars for the culture of *Rotylenchus robustus* on red clover or alfalfa seedlings. Nowadays, tissue culture laboratory ware, such as Magenta® jars and other devices (Fig. 6.5C,D), can be used to grow plant-nematode systems that support whole model plants such as *Arabidopsis* (Fig. 6.6).

Fig. 6.5. Transparent containers for gnotobiotic cultures. **(A)** Boiling tube containing coarse substrate to grow potato chits. **(B)** Polystyrene universal container, Eppendorf snap-cap microcentrifuge tube, Magenta jars. **(C)** Eppendorf snap-cap microcentrifuge tube adapted to culture tomato on sand and rockwool. **(D)** Tomato roots. (Copyright Rothamsted Research Ltd.)

6.10.1 Seed pouches

Soil-less growth or germination pouches produce clean pure cultures of plant-parasitic nematodes, which are ideal for TEM/SEM, and have been used mainly to observe invasion and development of nematodes on roots (e.g. Robinson *et al*., 1988) and provide clean egg masses of *Meloidogyne*; Atamian *et al.* (2012) describes the procedure for pouch culture.

The following protocols to obtain egg masses of *Meloidogyne* using pouches are based on Rao *et al.* (2012) and N. von Mende (Rothamsted Research, UK, 1994, personal communication). Adzuki beans (*Vigna angularis*) or tomato seeds are soaked in warm water (*ca* 40°C) for 4 h and germinated on moist filter paper in Petri dishes at 27°C until the radicle is 2–3 cm long. A small slit (1.5 cm long) is cut in the top of each CYG™ germination pouch (Mega International, St. Paul, MN, USA) and up to three individual seedlings are inserted

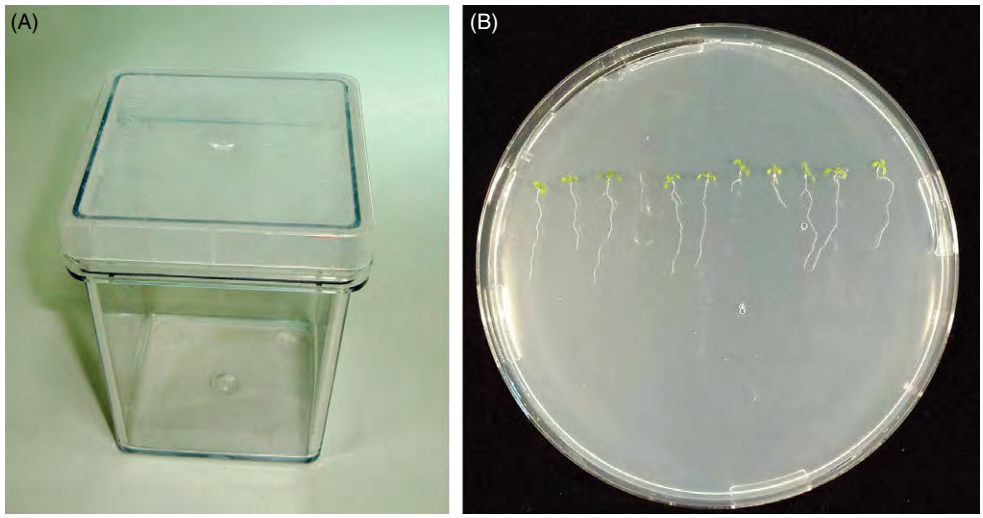

Fig. 6.6. **(A)** Magenta jar used to grow model plants such as *Arabidopsis thaliana* and *Lotus japonicus* filled with an appropriate substratum. (Photo: Rosa H. Manzanilla-López.) **(B)** *A. thaliana* culture. (Photo: courtesy of Federico López-Moya, University of Alicante, Spain.)

and stapled into the cut. The pouch is placed vertically in tap water within a plastic box with *ca* 2 cm of the brown paper of the pouch is immersed; the pouch is kept in a growth chamber for 2–3 days at 27°C to allow the roots to proliferate. Afterwards, cut the pouch down one or both sides (Fig. 6.7A–C), place a glass microfibre filter paper (Whatman, Maidstone, UK) under individual roots across the face of the brown paper, and inoculate freshly hatched J2 (300 in 100 µl of sterile dH_2O) with a micropipette (Fig. 6.7D) along the lateral root tip (Rao *et al.*, 2012) dripping the nematode suspension for about 1 cm up the root. Cover the inoculated area with a small piece of filter paper, smooth the side towards the root (i.e. roots are sandwiched between the two smooth surfaces of the paper). The pouches are separated and kept vertical by polystyrene sheets in plastic boxes (Fig. 6.7E). Plants are watered daily with diluted Hoagland's solution (1:500 stock solution) and kept at 27°C with 16:8 h light:dark (Fig. 6.7F).

The same procedure can be used to grow tomato seedlings (Fig. 6.7A–E) with some slight variations. Tomato seeds are incubated at 20°C for 5–6 days or until roots are 5–6 cm long. Roots are inoculated with J2 freshly hatched (2–4 days old maximum) at a concentration of 20–30 J2 in 10–20 µl dH_2O. Tape up the sides of the pouch and leave overnight at *ca* 20°C. After 24 h, remove the pieces of filter paper and wash the roots to remove any J2 that have not invaded (this is not necessary if an 'invasion cohort' is not required). Re-tape the pouch. Keep the pouches in a growth chamber in a large container of water so that the water level just reaches the brown tissue. One ml of full strength of Hoagland solution in 100 µl of sterile dH_2O can be added to 1000 ml of water and added instead of water to the pouches container. Plants can survive for 2 months after inoculation and females develop on the roots.

Pouches can work better for beans than for tomato seedlings as the latter may require extra nutrients and plants are more difficult to maintain in the glasshouse at 25°C for up to 8 weeks, the time when egg masses are ready to be collected (i.e. 6 and 8 weeks for adzuki beans and tomato, respectively).

6.11 Genetically Engineered Plants

Rhizobium rhizogenes-transformed roots ('hairy roots') grow rapidly, are highly branched, and tend to grow horizontally instead of downward. Transformed tomato roots are well adapted to axenic culture and their numerous lateral roots have proved suitable for culturing species of *Meloidogyne* (Verdejo *et al.*, 1988; Mitkowski and Abawi, 2002), *N. aberrans s.l.* (Manzanilla-López, 1997) and *H. schachtii*.

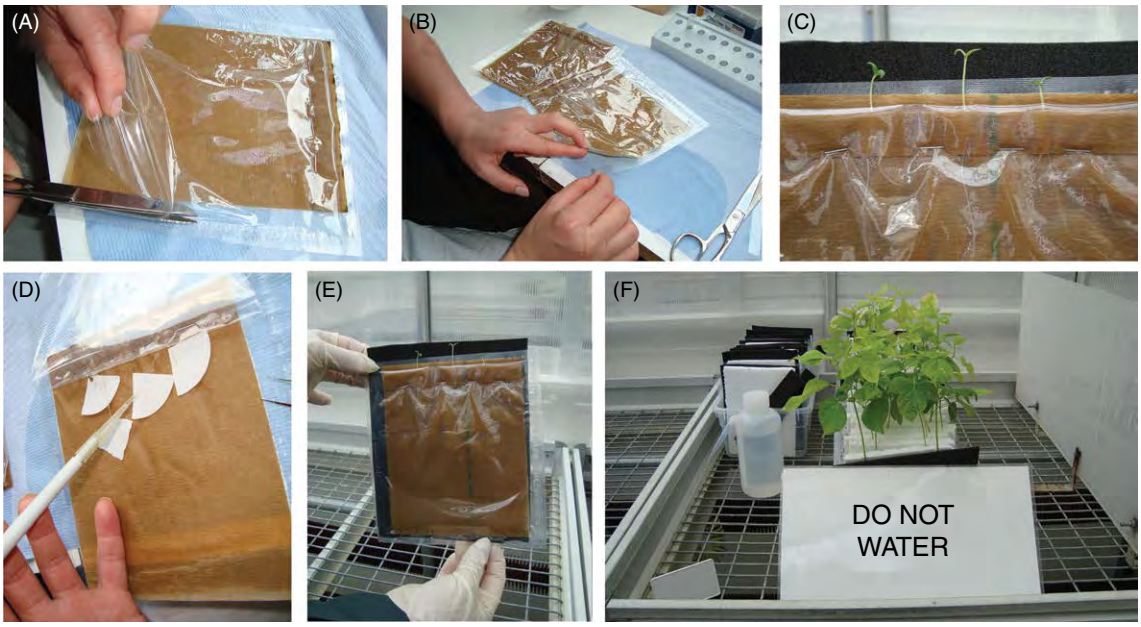

Fig. 6.7. Seed pouches for *Meloidogyne* spp. culture. **(A, B)** The pouch is cut down one or both sides. **(C)** Individual tomato seedlings are inserted and stapled into the small slit at the top. **(D)** Inoculation of second-stage juveniles on tomato roots placed onto glass microfibre filters. **(E, F)** Tomato and Aduki beans plants growing in pouches separated by polystyrene sheets in plastic boxes.

To culture *Meloidogyne* spp. (Verdejo *et al.*, 1988), roots genetically transformed with *R. rhizogenes* strain A4, were grown on Gamborg's B5 medium plus vitamins (Table 6.1) that was filter sterilized (0.22 µl pore) using 1 mg l^{-1} of calcium panthotenate, 1 mg l^{-1} nicotinic acid, 1 mg l^{-1} pyridoxine HCl, and 1 mg l^{-1} thiamine HCl; 10 mg l^{-1} of inositol and 0.01 mg l^{-1} biotin. Root pieces (2–3 cm long) from active growing cultures were transferred to new medium for 3–5 days before nematode inoculation (72 ± 8 eggs $plate^{-1}$). Eggs were obtained from egg masses produced on tomato monoxenic culture and were incubated for 4 min in 0.5 ml 0.525% NaOCl in a sterile conical centrifuge tube. The egg suspension was then diluted 20 times with sterile dH_2O and eggs that settled within 30 min were used as inoculum. Plates of each root culture were prepared and incubated in the dark at 25°C for 39 days. The agar was melted in a microwave oven and roots were removed.

Manzanilla-López (1997) cultured *N. aberrans s.l.* on transformed tomato roots. In a flow cabinet (previously sterilized with UV light), transformed tomato root sections (2–3 cm long) were cut with a sterile scalpel and placed with sterile fine forceps in Petri dishes containing Gamborg's B5 medium plus vitamins. The dishes were sealed with Parafilm and kept in the dark at 23°C for 2 weeks. Individual egg masses from galls produced on roots of glasshouse-cultured tomato plants were surface sterilized before inoculating into Petri dishes containing transformed tomato roots. Ten egg masses were adequate to inoculate a total of nine Petri dishes, each with an average of 300–700 eggs per plate. Plates were kept at 24.5°C for 3 months. The Bolivian population of *N. aberrans s.l.* was able to develop from J2 to egg in 60 days.

A protocol for culturing *H. schachtii* on sugar beet roots transformed with *R. rhizogenes* was described by Paul *et al.* (1987). J2 were surface sterilized by pouring a suspension onto a Sartorious membrane filter (5 µm pore diam.) and washed with a 0.02% (w/v) $HgCl_2$ solution for at least 3 min, and then extensively washed with sterile dH_2O. The J2 were re-suspended in a 0.05% (w/v) tetracycline solution for at least 15 min. Small droplets of the J2 suspension were deposited with a micropipette on the roots in the Petri dishes. After inoculation, the plates were sealed with Parafilm and stored at 22°C. Hairy root cultures were suitable for the induction of syncytia and development of *H. schachtii* and the nematode did not lose its pathogenicity after being cultured.

6.12 Model Plants

Arabidopsis thaliana has been the major plant model system in the past three decades for research on, for example, plant development, signalling, hormone biology, pathogen defence, disease resistance and abiotic stress response. Model plants capable of nitrogen fixation include *Medicago truncatula* and *Lotus japonicus* (Fig. 6.8). Other common model plants include tomato that can be infected by a large number of pathogens; tomato is also easily transformable and the sequencing of its genome has strengthening its position as a model for plant–pathogen interactions (Piquerez *et al.*, 2014).

6.12.1 *Arabidopsis thaliana*

Arabidopsis thaliana (Fig. 6.6B) is able to complete its life cycle in a Petri dish, thus providing a miniature plant system with transparent roots successfully used in research on life cycle and behaviour of various species of nematodes, including *Helicotylenchus multicinctus* (Orion and Bar-Eyal, 1995) and cyst (e.g. *H. schachtii*, *H. trifolii*) and root-knot nematodes (e.g. *M. javanica*, *M. incognita*). Transformation of *A. thaliana* (ecotype C24) carrying the GUS reporter gene was made by Goddijn *et al.* (1993), which has enabled the observation of plant gene regulation in nematode feeding sites. Research on plant-nematode interactions and other micro-organisms using *A. thaliana* has increased notably, especially in relation to omics approaches (e.g. Huang *et al.*, 2006; Barcala *et al.*, 2010; López-Moya *et al.*, 2017).

Several protocols exist to surface sterilise seeds of *A. thaliana*; the following protocol is from N. von Mende (Rothamsted Research, UK, 1994, personal communication):

- Prepare fresh sterilization solution:
  - 1 ml Teepol (0.8% available chlorine = final concentration)
  - 4 ml dH_2O
  - 1 drop Tween 20.
- Place small volume of seeds (*ca* 50 µl) in an Eppendorf tube and add 1 ml 70% ethanol for 1 min.
- Discard ethanol and add 1 ml sterilization solution.
- Over a 5 min period re-suspend the seeds several times.
- Discard the supernatant and wash at least five times with sterile dH_2O.
- For individual seed transfer, pipette the seeds on to sterile filter paper and use fine sterilized forceps to transfer them to agar plates containing Knop medium (Table 6.4).

Table 6.4. Knop medium.

Solution number	Stock solution	Quantities (g l^{-1})
I	KNO_3	121.32
	$MgSO_4.7H_2O$	19.712
II	$Ca(NO_3).4H_2O$	120.0
III	KH_2PO_4	27.22
IV	NaFeEDTA	7.34
V	H_3BO_3	2.86
	$MnCl_2.4H_2O$	2.85
	$CuSO_4.5H_2O$	0.073
	$ZnSO_4.7H_2O$	0.360
	$CoCl_2.6H_2O$	0.030
	NaCl	2.0

Add the following volumes from solutions into a 1 l flask:
- 2 ml solution I; 2 ml II; 0.4 ml III; 0.4 ml IV; 0.2 ml V.
- Add 1.5% sucrose (15 g l^{-1}) and adjust to pH 5.8–6.0, make up to 1 l with dH_2O.
- Add 0.8% Daiching agar and then autoclave. Let it cool slightly and add 1 ml vitamin solution (Gamborg's) and pour into plates.

- The plates were placed in a constant temperature room at 23°C with a 16.5:7.5 h light:dark cycle. Once seedlings were produced (4 to 5 days) and the root system exhibited lateral roots, plates can be inoculated.

Sijmons *et al.* (1991) optimized the culture conditions for the infection of seedling of *A. thaliana* with *H. schachtii*, *M. arenaria* and *M. incognita* using a modified Knop's medium. Shah *et al.* (2017) grew *Arabidopsis* plants in either Knop's medium for *H. schachtii* infection or Murashige and Skoog medium for root-knot nematodes infection. Mendy *et al.* (2017) inoculated 12-day-old *Arabidopsis* plants with 60–70 J2 of *H. schachtii* in agar medium supplemented with modified Knop's nutrient medium under sterile conditions. Barcala *et al.* (2010) sowed an average of ten surface-sterilized seeds of *A. thaliana* in modified Gamborg's B5. Plates were kept at 4°C for 2 days, and transferred to a growth chamber at 25–26°C, 60% relative humidity and long day photoperiod. Four days later, each root tip was inoculated with 10–12 freshly hatched *M. javanica* J2. López-Moya *et al.* (2017) surface-sterilized seed using 1% NaOCl for 2 min, then washed seeds three times with sterile dH_2O. Afterwards seeds were stratified at 4°C for 48 h and then grown on Murashige and Skoog medium plates.

Goddijn *et al.* (1993) transferred 10-day-old seedlings to potting soil in 10 × 30 × 100 mm translucent containers that were put at an angle of 45°C in trays and incubated in growth rooms (16:8 h, light:dark; 22°C). Niebel *et al.* (1994) developed a hydroponic inoculation system where *A. thaliana* was grown in glasshouse conditions on sand and supplemented with a nutrient solution to facilitate scoring nematode infection after rinsing the roots in water. Plants were inoculated 7–14 days after germination, and produced up to 130 females when inoculated with 1000 J2 $plant^{-1}$.

6.12.2 *Lotus japonicus*

Most crop plants are grasses (monocotyledon), and many others are legumes that fix nitrogen from the air. *Lotus japonicus* is one of the most useful plants for legume study and is amenable to plant transformation and regeneration from tissue culture. Calli can be obtained from hypocotyls, leaves, roots and petals cultivated on Gamborg's B5 medium containing 2,4-D and kinetin (Piquerez *et al.*, 2014). *Lotus* would greatly facilitate a better understanding of the differences between parasitic (nematode) and mutualistic (rhizobia and mycorrhizae) symbiosis.

Seeds are scarified and sterilized in concentrated sulphuric acid, washed with sterile dH_2O (Lohar and Bird, 2003; Cabrera Poch *et al.*, 2007) and germinated on wet filter papers before sowing them on agar Murashige and Skoog medium (Cabrera Poch *et al.*, 2007). After germination seedlings can be transferred to pots or plants can be grown on plates under sterile conditions on plant-growth medium agar, keeping the roots in darkness. Alternatively, seedlings can be grown in Magenta boxes filled with vermiculite or perlite:vermiculite (Pajuelo and Stougaard, 2005), plastic pots containing a mixture of 1:1 v/v sand and peat moss (Cabrera Poch *et al.*, 2007), fine vermiculite, clay litter, or a 1/1, v/v mixture of river bottom sand and peat (Lohar and Bird, 2003).

Fig. 6.8. *Lotus japonicus*. **(A)** Individual plant. **(B)** Glasshouse culture of different accessions of the plant.

Lotus can also be cultured hydroponically, where seeds are placed on a mesh and submerged in plant-growth solution, and aeroponically, where plant-growth solution is misted around the roots (Pajuelo and Stougaard, 2005). Features of *Lotus* biology, such as thin and translucent roots, have proved ideal for monitoring the progress of *M. incognita* and *H. glycines* infection both on live specimens and post-stained specimens. Lohar and Bird (2003) examined *L. japonicus* mutants (i.e. a transgenic line for the *A. thaliana etr-1* mutant allele of *ETR1*) with nodulation phenotypes and considered *L. japonicus* as a powerful model legume for studying compatible and incompatible plant–nematode interactions. Amin *et al.* (2014) produced a system for quantitative analysis of all stages of *Meloidogyne* infestation on *L. japonicus*. They also produced a root transformation protocol for *L. japonicus* to facilitate downstream molecular studies, including specific high-throughput screening of nematode resistance traits.

6.13 Pot Cultures

Conventional glasshouse culture techniques, e.g. using an appropriate substrate, susceptible plants in pots or microtanks (thermostatically controlled water tanks), require considerable space and careful management of factors that affect nematode invasion and multiplication, including plant age, relative humidity, and water temperature and depth. Soil-less substrates include combinations of minerals, synthetic and organic substances, some of which can be used in pot cultures of migratory endoparasitic nematodes (De Ley and Mundo-Ocampo, 2004) such as *Radopholus similis* in *Anthurium andraeanum* (Wang *et al.*, 1997). To produce and maintain *M. graminicola* cultures, Kumar *et al.* (2017) developed a laboratory/growth room technique based on a soil-less system that ensures continuous availability of large amounts of nematode inoculum.

Avoid cross-contamination by wearing gloves when handling cultures and change them every time you are working with a different species or population. Ensure disinfected scissors and tweezers are used when handling root systems. Label pots and cultures with permanent ink or pencil.

6.13.1 Pot cultures of *Meloidogyne* and *Nacobbus*

Before starting a culture check the species; host-specific species of *Meloidogyne* must be cultured on their particular host. Non-host-specific populations can be cultured on a susceptible variety of tomato (e.g. 'Moneymaker'), aubergine (*Solanum melongena*) or Busy Lizzie (*Impatiens walleriana*). Grow the host plant to the first leaf stage (12–15 cm high for tomato seedlings) and transplant into a pot filled with sterile compost (add slow release fertilizer), leaving a reasonable distance between soil and the top edge of the pot to avoid splash contamination between neighbouring pots. Plants can be inoculated 2 or 3 days later, allowing the seedling to establish and grow. *Meloidogyne* and *Nacobbus* glasshouse cultures should be started with J2 from a single egg mass to ensure con–specificity.

Egg masses are cleaned and eggs left to incubate to produce J2 in order to start fresh, non-contaminated, cultures. To obtain free eggs from the egg masses and hatched J2 see Perry (Chapter 9, this volume). Dampen the soil slightly prior to inoculation. Using a Pasteur pipette inoculate plants with a suspension of 1000–5000 extracted eggs or hatched J2 (surface sterilized) into equidistant holes (3 cm depth) angled under the plant and close to the base of the plant. Holes are subsequently filled with soil and pots are given a light watering. Numbers of eggs or J2 can be reduced according to pot size and inoculum availability (a general rule is 2 nematodes (g soil)$^{-1}$).

Once nematodes are producing egg masses, subcultures can be established. Prepare infested soil in a plastic bag (e.g. an autoclave bag) by thoroughly mixing compost with half or part of the infested soil (and root pieces) contained in the original pot. Fill new pots with the mix and transplant new seedlings into these pots. Subculture regularly – tomatoes every 3 months or as needed – or they can be pruned regularly to reduce the need for sub-culturing. Perennials generally require less frequent re-potting/re-seeding and it may not even be necessary.

Ensure the temperature regime matches the nematode's requirement, especially for *Meloidogyne* species. Most temperate species can be kept at 18–22°C and tropical or sub-tropical species at 25–30°C. Do not overwater. If watering is done by pouring water into the bottom of a tray, avoid putting pots containing

different nematode species in the tray; ideally pots with different species should be kept separated by Perspex® screens to avoid cross-contamination by splashing.

Tomato flowering generally coincides with the onset of egg production by *Meloidogyne* and *Nacobbus* females. Tomato plants should be checked soon after flowering (*ca* 15–20 days after transplanting) for the first egg masses. Plants are removed from the pots, put in polythene bags and transferred to the laboratory where the soil is gently removed from the roots and the roots submerged in a beaker of water (1 l). Roots are wrapped in moistened 'kitchen towel' paper and placed inside a plastic bag in the refrigerator at 5°C for 5 days, after which they are taken out, excess water is removed and cut into 2–3 cm pieces. The pieces are place in water in a Petri dish (11 cm diam.) and examined under a dissecting microscope for the egg masses.

Nacobbus aberrans occurs in soil not only as eggs and egg masses as in root-knot nematodes, but also as juvenile stages and young vermiform adults. Detection of *N. aberrans* in soil samples can be compromised due to the resting stages in soil, which need to be re-activated in order to produce galls. This re-activation can be made through bioassays which provide appropriate conditions of humidity, temperature and a suitable host. *Nacobbus aberrans* closed plastic bag method detects the nematode even in slightly infested soils (Atkins *et al*., 2005; Ortuño *et al*., 2005).

- Homogenize the infested soil sample, take a 400 g subsample and transfer 200 g into a transparent polyethylene (plastic) bag (10 × 20 cm)* Water soil with 50 ml of water in order to provide uniform moisture (field capacity).
- Sow a clean sprouted potato chit.
- Close the bag by bending at the top, staple and incubate* in the dark at 25°C for 30 days.
- After 30 days of incubation look for the presence of galls as they can be seen directly in the soil sample through the plastic. If galls are not evident, leave the sample for another 10 days.

*Notes: A re-sealable plastic bag (18 cm × 20 cm) can be used and a clear plastic tube inserted into each bag to provide aeration and the bag labelled and sealed (Fig. 6.9). If an incubator is not available, cardboard boxes lined (base, sides and top) with six layers of newspaper can be used.

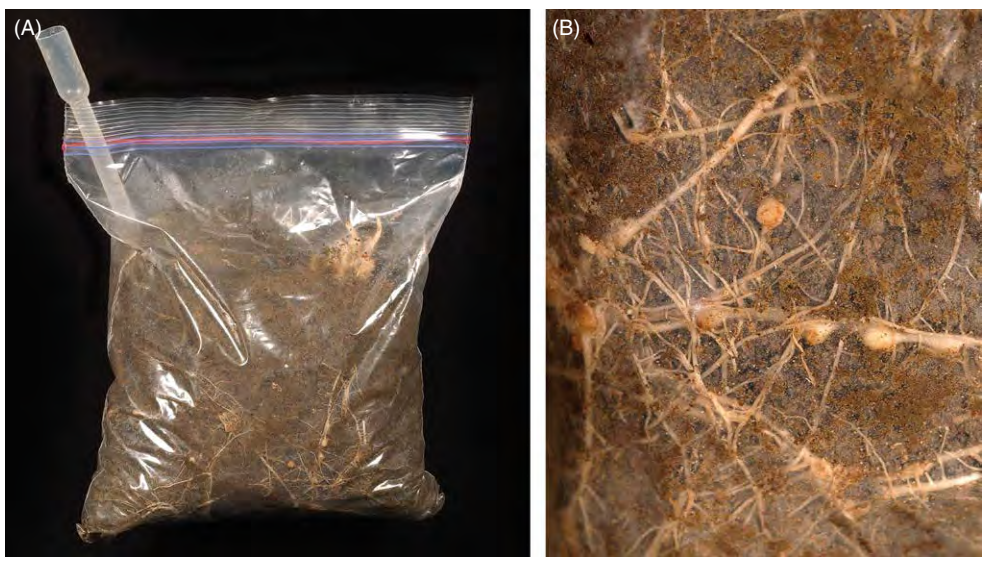

Fig. 6.9. *Nacobbus aberrans* closed plastic bag bioassay. **(A)** Re-sealable plastic bag and plastic tube inserted to provide aeration. **(B)** *N. aberrans* galls. (Images after Atkins *et al*., 2005.)

6.13.2 Pot cultures of cyst nematodes

Pot culturing cyst nematodes is a standard method of obtaining large quantities of cysts and can be used to obtain cysts representing a single generation. For *G. rostochiensis* and *G. pallida* diapause can delay hatching; for details of diapause, methods for collecting and using host root diffusate to obtain J2, and a culture technique to avoid the establishment of diapause in cultures see Perry (Chapter 9, this volume).

The basic method of culturing cyst nematodes is similar for all species; differences are in the type of plant material used, e.g. potato pieces for cultures of *G. rostochiensis* and *G. pallida* and seeds for *H. schachtii* but extraction methods and storage conditions differ among the two genera. The following description is for pot culturing of *G. rostochiensis* and *G. pallida* in 15 cm diam. clay pots. A 3:1 mixture of loam and sand should be used as the soil base, preferably steam sterilized to ensure it is free from other pathogens. Peat should not be added as it impedes cyst recovery. Pure sand can be used but plants must be fed regularly with a nutrient solution. To start a culture 3000–5000 eggs or J2 are placed in each pot, or alternatively 20–30 cysts if they are single generation cysts of up to a year old (cysts extracted from cultures can be stored dry at ambient laboratory temperature, but will need rehydrating in tap water before use for experimentation or sub-culturing). The age of inoculum is important: 3-year-old inoculum, use 30 cysts pot^{-1}; 5-year-old inoculum, use 40 cysts pot^{-1}; 8-year-old inoculum, use 60 cysts pot^{-1}; 10-year-old inoculum, use 75 cysts pot^{-1}; older inoculum, use 100+ cysts pot^{-1} (M. Russell, Rothamsted Research, UK, 1994, personal communication).

Seed tubers will need to be set to chit approximately 3 weeks in advance of use, in a well-lit store at 10°C. Set out sufficient tubers on the assumption that each will develop only three useable sprouts. Use a single-sprout piece of potato tuber for a 10 cm diam. pot, choosing sprouts about 2.5 cm long with several root initials. 'Desiree' and 'Maris Piper' are usually used for *G. rostochiensis* and *G. pallida*, respectively. For standard culturing, the cyst inoculum can be added loose to the soil beneath the potato chit. However, for experiments where hatching percentage is required, it is best to enclose the cysts in a bag made of a small square of polyester voile (mesh small enough to retain cysts but large enough for J2 egress) and secured with polyester thread. Procedure:

- Fill the 15 cm diam. clay pot one third with growing medium.
- Cut sprouts from the chitted potato seed tubers, leaving a piece of potato tuber beneath the sprout about 2 cm in depth.
- Place the bagged cysts on the soil surface or scatter loose cysts on the soil surface and then place a potato chit on the soil surface.
- Fill the pot with soil to the base of the rim, add a label to record population details and sowing date; add a small pinch of slow release fertiliser to the soil surface.
- Place the pot in the glasshouse and water normally; keep pots of each species and population separated to prevent cross-contamination.
- Plants should be left for a minimum of 12 weeks or until senescence; if females are required at the white stage, they can be picked directly from the roots from about 7 weeks after planting; such females will not be virgin (for methods to obtain males, and virgin females and associated sex pheromones see Perry, Chapter 9, this volume).
- After the plant has senesced (or after 12 weeks), cease watering, and remove the plant top. To speed drying of the soil, the pot can be transferred to a drying cabinet at 25–30°C, but no warmer or the cyst contents may be killed. Cysts can then be extracted from the dry soil (see Viaene *et al.*, Chapter 2, this volume).

For glasshouse cultures of *H. trifolii* (Mercer, 1990; Mercer and Grant, 1993) cysts are obtained from infested soil and infected clover roots by washing over nested 2 mm, 600 µm and 180 µm sieves, and extracted from the fine soil on the 180 µm sieve by centrifugation in sugar solution (Viaene *et al.*, Chapter 2, this volume). After rinsing in running tap water on a 150 µm sieve, cysts are broken open to release the eggs by using a rubber food scraper drawn over the mesh. Host plants are inoculated 2 weeks after sowing (2200 eggs $plant^{-1}$). If collected from infested field sites, half of the soil containing the nematode, should be mixed with a similar volume of sand/soil mix, put into pots and sown with white clover seed (Mercer and Grant, 1993). The egg suspension is inoculated into a hole angled under the plant using a Pasteur pipette.

Singh and Norton (1970) cultured populations of *H. trifolii* from single cysts on a susceptible white clover (*Trifolium*; 'Ladino') under glasshouse conditions. Plants were grown at 24–26°C and cysts were harvested 80 to 90 days later. In an alternative method, Singh and Norton (1970) germinated seeds on wet blotting paper in a Petri dish and, after emergence of the radicle, the seedlings were transferred to moist, fine sand in another Petri dishes. Forty-eight hours later, 200 washed J2 were poured around the roots in each dish and after a further 24 h seedlings were carefully lifted, washed to remove any unattached J2, and transplanted to pots.

6.13.3 Storage of viable cysts of *Heterodera* spp.

Large-scale glasshouse pot experiments to screen resistance/susceptibility to *Heterodera* spp. will require a large number of cysts, thus the importance not only of harvesting but storing viable cysts. Chapman and Eason (1973) placed several hundred cysts of *H. trifolii* in a 90 mm diam. glass Petri dish, then covered with a thick layer of molten, cooled 5% agar. The dish(es) were placed in freezer plastic bags and stored in a refrigerator at 10°C. Cysts kept in 5% agar can be viable up to 18 months. If the agar becomes dry and brittle, pieces can be placed in a mist chamber to recover juveniles. Freshly extracted cysts of *H. glycines* can be placed on a slightly moistened filter paper folded to form a small envelope and wrapped with cling film. The envelope is placed inside a small plastic container with a lid and kept at 4°C in a refrigerator. Cysts remain clean and viable for at least 1 month.

6.14 Production of Entomopathogenic Nematodes

6.14.1 *In vivo* culturing

6.14.1.1 Production of the host insect, Galleria mellonella

Most entomopathogenic nematodes (EPN) reproduce well in the lepidopteran larvae of the greater wax moth, *Galleria mellonella*. Other lepidopterans, like *Bombyx mori* (silkworm) or *Manduca sexta* (tomato hornworm), can also be used and coleopteran mealworms, *Tenebrio molitor*, also provide good EPN yields. If *in vivo* reproduction is not successful and the natural host insect is not known, *in vitro* culture is recommended.

As *G. mellonella* is the most frequently used insect for rearing EPN, the propagation of the insect is explained here. This insect can be reared at high density as they are not cannibalistic like some other lepidopterans. The cycle from egg to last instar lasts approximately 5 weeks at 25°C and is even shorter at 30°C. One or two additional weeks are needed to obtain adult moths. The natural environments of *G. mellonella* are beehives, where they consume the wax. Hence, the natural medium is the honeycomb, which can be purchased from beekeepers and often even contain some larvae.

An artificial *G. mellonella* culture medium contains wheat flour 15%, polenta 15%, honey 20%, glycerin 15%, milk powder 10%, soy flour 10% and yeast flakes 15%. Avoid the use of transgenic corn, which contains entomopathogenic *Bacillus thuringiensis* Cry1 toxins that will kill *G. mellonella* larvae. The addition of beeswax (approximately 10%) will improve the quality of the wax moth larvae. Mix honey, glycerin and yeast powder, then add cereals and milk powder. If beeswax is added, melt it separately at 80°C and then add the liquid wax to the mixture. If fungal growth in the medium is a problem, 0.5% Nipagin (p-hydroxybenzoic acid methyl ester) can be added to the medium.

Culture between 20 and 30°C and, because *G. mellonella* larvae easily bore holes into plastic ware, use metal or glass containers covered with a metal mesh to allow aeration. Females lay eggs onto filter paper, which can be placed under or above the metal mesh or added to the culture jar. Eggs on filter paper are transferred to fresh medium and larvae hatch after about 3 days. Only the bottom of culture containers should be covered with medium and as larvae develop fresh medium should be added weekly. When larvae reach the sixth stage before pupation, they stop feeding, move to the top of the rearing device and produce a silk cocoon, where they remain for approximately 2 weeks before adults emerge. Adults do not need food or drink.

The *in vivo* reproduction of EPN on insects is done by infecting insects with the nematodes. Petri dishes (9 cm diam.) lined with moist filter paper or filled with sand of 10–15% water content are inoculated with approximately 20 *G. mellonella* last instar larvae. Add approximately 100 dauer juveniles (DJ) per insect, ensuring that a minimum amount of water, enough to moisten the filter paper, is transferred with the nematodes; infection is low if too wet. Seal the lid with Parafilm and store at room temperature in the dark. There is no need to add water. After 10 days, transfer the dead insects into a water trap (Ehlers and Shapiro-Ilan, 2005). Put the bottom part of the Petri dish with the insects into a larger dish filled with 2 mm of water. Nematode DJ will exit the cadavers, migrate over the filter paper, climb over the rim of the small dish and end up in the water. Harvest the water containing the DJ into a beaker every 2 days after the emigration has started, and replace the water in the trap. Add 0.01% formalin to the water in the trap if there are too many contaminants and infestation with scavenger insects occurs (frequent in tropical climates). After 12 days the emigration ceases.

To clean the DJ suspension, pour the contents of the beaker over a sieve (50 µm mesh). If the DJ hit the sieve 'head first' they will pass through, so to minimize loss incline the sieve by 45° so the majority will slip off the mesh and not pass through. If only a very few DJ are available, and any loss cannot be afforded, use a 5 µm sieve because the DJ cannot pass through, even head first. Transfer the clean suspension into tissue culture flasks to a maximum depth of 5 mm. Use flasks with vented caps to allow air exchange and store flasks at 4–15°C, depending on nematode species and their tolerance to low temperature. Clean the suspension by removing dead nematodes every 2 weeks. The Baermann funnel extraction technique (see Viaene *et al.*, Chapter 2, this volume), used for 1 h, can also be used to separate living from dead DJ. In most laboratories, several strains or species are handled at the same time. To avoid cross-contamination, wash instruments with hot water (> 60°C) before using them for another strain. Plastic and glassware can be stored in ethanol.

6.14.1.2 Counting dauer juveniles

For smaller units suspend DJ in 5 ml water, shake well and take three samples of 100 µl. Count the DJ in each sample and calculate the mean of the three samples; multiply by 50 to give the total amount in 5 ml.

To count nematodes in a commercial formulation (e.g. 50×10^6):

- Open package and pour contents into 5 l tap water at 10–20°C.
- Stir suspension vigorously for 1 min.
- Keep agitated by bubbling air in from a tube leading to the bottom of the bucket or continue to stir frequently.
- Take 3 × 100 µl samples into three clean test tubes (use pipette with < 1% error).
- Add 4.9 ml tap water to each test tube (use glass-pipette with < 2% error).
- Mix suspension by shaking tubes and immediately (< 1 s) after shaking take 5 × 100 µl aliquots from each tube.
- Place in clean Petri dishes and count living nematodes in the droplets using a dissecting microscope with >40× magnification.

There are always some dead nematodes in any formulation or storage stock. To distinguish living from dead nematodes use the following characters: living DJ move, resting individuals are never completely straight. Search for coiling or DJ with at least the head or tail bent. Nematodes usually start moving slowly after suspension in water. DJ with a straight body shape filled with vacuoles, and a shrivelled, uneven surface are dead.

6.14.2 *In vitro* culturing

The insect's defence mechanisms eliminate contaminating bacteria in the haemocoel after invasion of the DJ. Once the host insect dies, the gut microbiome colonizes the insect but nematode reproduction has usually started. When culturing *in vitro*, sterile handling is an absolute pre-requisite because anything other than monoxenic cultures (nematode and its symbiotic bacterium only) will fail. The first step to culture *in vitro* is to produce a pure culture of the symbiotic bacterium. Such a bacterial culture is then inoculated with bacteria-free nematodes.

6.14.2.1 Isolation of symbiotic bacteria and production of bacterial stock cultures

The symbiotic bacteria *Xenorhabdus* or *Photorhabdus* spp. are isolated from larvae of *G. mellonella*. On invasion of the insect by the DJ, non-symbiotic bacteria invade the haemolymph, but the insect's defence system will usually eliminate them as long as only a few nematodes are used to infect the insects.

- Infect each insect with approximately 10 DJ from *G. mellonella* (as described above). Use sand for *Heterorhabditis* (10% moisture). Do not use too many DJ; they will destroy the intestine or overcome the defence reaction too soon and then contaminants that leak into the haemolymph may establish.
- When the larvae are moribund, cut a proleg and streak a drop of exuding haemolymph on Nutrient Bromothymol blue Triphenyltetrazolium chloride Agar (NBTA). Best results are obtained when the insect is still alive with hardly any contaminating bacteria.
- Incubate at 25–30°C for 2 days. Whatever grows after 1 day is a contaminant; colonies of the symbiotic bacteria need 2 days to be visible. Colonies of the symbiotic bacteria absorb the bromothymol blue (BTB) colour and can easily be distinguished from other bacteria.
- To produce NBTA, add 25 mg l^{-1} of BTB to any kind of bacterial standard NA and autoclave.
- When cooled to 50°C add 4 ml of a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC). This component is heat labile. Therefore, it must be filter sterilized and then added to the medium.
- TTC produces a red colour under reductive conditions and will produce red colonies with contaminating bacteria and secondary symbiotic bacteria, which do not effectively support nematode reproduction (Akhurst, 1980).
- Pick a single colony and transfer into 20 ml Yeast Salt Medium and incubate at 25–30°C on a shaker in the dark for 24 h.
- To produce YS broth, mix 5.0 g yeast extract, 5.0 g NaCl, 0.5 g $NH_4H_2PO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \times 7\ H_2O$ with 1 l dH_2O.
- From this culture, stocks can be produced by preserving 15% glycerin suspensions of the incubated YS culture in Eppendorf caps at –35°C or –80°C.

6.14.2.2 Identification of contaminants and phase variants of symbiotic bacteria

Monoxenic cultures of EPN need to be free from any kind of contaminating microorganisms, otherwise they produce very few offspring and later fail when sub-cultured. Distinguishing the symbionts from other bacteria is not easy and needs experience. Cell morphology and colony morphology are variable. The task is further complicated because of the phase variation of the symbiotic bacteria. The so-called primary form, isolated from DJ, can shift to the secondary form, which does not effectively support reproduction of the nematodes. The shift is a response to stress and consumption of the medium compounds. Secondary form bacteria are also found in insect cadavers at the time the DJ emigrate. The shift is not a 100% change and both colony forms and intermediate forms are found. The secondary form loses the typical characters of the primary form and thus can often be interpreted as contaminating bacteria. Typical biochemical characters used to describe the metabolism of bacteria (e.g. metabolization of carbohydrates) are usually highly variable and thus cannot serve to distinguish the forms. Table 6.5 provides some information for identification of primary and secondary form (phase variants) of *Xenorhabdus* and *Photorhabdus* spp.

6.14.2.3 Production of bacteria-free nematodes

Early publications recommended the use of surface-sterilized DJ to inoculate the symbiont cultures. Although DJ may only carry the bacterial symbionts in their gut, non-symbiotic bacteria can survive on the outside of the DJ or between the pre-dauer and dauer sheath. Success of the sterilization process cannot be guaranteed. This disadvantage can be overcome by sterilization of nematode eggs according to Lunau *et al.* (1993). Eggs are isolated and sterilized and hatching first-stage juveniles (J1) can be checked for the presence of contaminants before they are combined with the bacterial cultures.

Table 6.5. Characters of primary and secondary form bacteria of *Photorhabdus* and *Xenorhabdus* spp.

Investigation of	Primary form	Secondary form
Colony morphology	Circular, convex, granulated and opaque with irregular margin, mucoid and sticky	Flat, translucent with regular margin and usually greater diameter, easily soluble in water
Colonies on McConkey-Agar (Akhurst, 1980)	Colonies absorb Neutral Red: red- or pink-coloured colonies	No absorption of Neutral Red: white or yellow colonies
Colonies on NBTA-Agar (Akhurst, 1980)	After 3–5 days, colonies absorb BTB: blue, olive or green colour, clear yellow zone around colony, where agar is partly discoloured	No absorption of BTB: red or maroon colonies without a clear zone, blue colour at margin of colony
Pigmentation in liquid culture	Depends on strain and medium. *Photorhabdus* spp. cultures are orange to brick red and change colour to violet-purple when concentrated sodium hydroxide is added	Depends on strain and medium. Usually more yellowish or orange than red. Colour change is less pronounced after adding NaOH, due to low pigment concentrations
Cell shape and morphology	Small- to middle-sized rod-shaped cells (3–5 µm long, 1.5–2 µm wide), majority with ovoid and/or rhomboid-rectangular inclusion bodies	In some species relatively long cells (6-7 µm long, sometimes longer, 1-1.5 µm wide), inclusion bodies rarely found
Bioluminescense (only *Photorhabdus*)	Positive, 72-h-old colonies can be identified in the dark after 5 min to allow for adaptation of the eyes	Negative
Antibiotic activity in overlay agar[a]	Positive	Negative
Nematode growth in bacterial culture	Good propagation of nematodes, fast increase in size, high number of offspring and good survival	Hardly any propagation of nematodes, stagnating growth, low number of offspring, high mortality

[a]Grow bacteria (*Photorhabdus* or *Xenorhabdus* spp.) in YS medium for 24 h. Glass dishes with nutrient agar are point-inoculated with six drops of 10 µl symbiont bacteria solution to obtain six colonies of 1 cm diam. and are incubated for 48 h. Dishes are then exposed to chloroform for 2 h and after evaporation of the chloroform, a 50°C nutrient agar mixed with *Bacillus cereus* is overlain. Two days later the growth inhibition zone of *B. cereus* can be evaluated.
NBTA = Nutrient Bromothymol blue Triphenyltetrazolium chloride Agar; BTB = Bromothymol Blue

- Infect each *G. mellonella* last instar larvae with about 100 DJ (*Heterorhabditis* spp.) or 50 DJ (*Steinernema* spp.) and keep at room temperature. Incubation of insects in 10% moist sand increases infection.
- Dissect dead insect larvae 3–6 days after infection and collect gravid females (*Steinernema*) or hermaphrodites (*Heterorhabditis*) into a staining block filled with Ringer's solution, containing 9.0 g NaCl, 0.42 g KCl, 0.37 g $CaCl_2 \times 2\ H_2O$, 0.2 g $NaHCO_3$ in 1 l dH_2O. The best time to collect the nematodes is when egg laying has started, but *endotokia matricida* (Johnigk and Ehlers, 1999) should not have begun yet. At this stage there are many fertilised eggs in the uterus. At least 100 large adults are necessary for successful egg isolation.
- Wash nematodes until no more debris and pieces of the insects remain. Smaller nematode stages like J1 and second-stage juveniles (J2) should be removed.
- Transfer the gravid adults into a glass tube.
- Cut the sharp edge of razor blades into 2–3 mm long pieces and add them to the nematodes.
- Vortex to cut adults and release eggs; continue until only small pieces of the adults remain and the solution is turbid.
- If necessary, debris can be removed by passing eggs through a 50 µm sieve.
- Transfer eggs into an Eppendorf tube (2 ml) and centrifuge for 1 min at low speed to sediment the eggs.
- Remove supernatant carefully, retaining the eggs, and add fresh Ringer's solution and repeat centrifugation. Wash again until supernatant remains clear.

- Add 1 ml of surface sterilization solution, containing 0.5 ml NaOCl (12%), 1.5 ml 4 M NaOH (160 g to 1 l dH_2O) and 10 ml dH_2O.
- Shake gently for 4 min, then centrifuge 2 min at low speed; do not go beyond 6 min sterilization time, otherwise too many eggs will die. Embryos can also be damaged by too high a centrifugation speed.
- Remove the supernatant with a sterile pipette; fill with sterile phosphate buffer or YS broth.
- Centrifuge again. Repeat washing with YS once more. Addition of glucose (1 g l^{-1}) to the YS broth prolongs survival of the hatching J1.
- Transfer eggs to sterile cell wells (24 well plates) filled with 300 µl YS medium.
- Incubate for 72 h. Growth of microorganisms (contaminants) is visible by increased turbidity of the YS broth.

Crucial for the success of this method is the isolation of gravid adults at the right moment and sterile handling after surface sterilization of the eggs. Adult female nematodes can also be collected from monoxenic cultures (see below).

6.14.3 Establishment of monoxenic cultures

- Two days after egg isolation, inoculate YS medium (20 ml in l00 ml Erlenmeyer flask) with the primary form of *Xenorhabdus* or *Photorhabdus* isolated from the corresponding nematode strain.
- Incubate culture on a shaker at 200 rpm and 25°C in the dark for 24 h.
- Prepare Nematode Growth Agar (NGA), containing 15 g agar, 2 g peptone, 2 g yeast extract, 4 g NaCl, 0.35 g KCl, 0.3 g $CaCl_2$, 0.2 g $MgSO_4 \times 7\ H_2O$ and 5 µg l^{-1} cholesterol (1 ml of a 5 mg ml^{-1} solution in ethanol) in 1 l dH_2O.
- Inoculate 6 cm diam. Petri dish with NGA with two drops of bacteria suspension from YS broth and 50–100 J1 from sterile cell wells in a minimum of water. When J1 swim on the agar surface, monoxenic cultures are usually not successful.
- Close plates with Parafilm and incubate in the dark at 25°C.
- Check for nematode development daily.
- After 5 days, take sample with a bacterial loop and streak on NBTA and incubate at 30°C to check for contaminants.
- If the culture is monoxenic, the resulting DJ can be used to inoculate subsequent cultures.
- Monoxenic cultures can be stored at 4–15°C.

6.14.3.1 Monoxenic cultures for mass production

Monoxenic cultures started from eggs should be sub-cultured a few times on Wouts Agar, containing 12 g nutrient broth, 12 g agar and 5 g vegetable oil in 1 l water. These cultures can then be used to inoculate cultures with solid medium in plastic bags (Bedding, 1984) or transferred to liquid cultures (Ehlers *et al.*, 1998; Ehlers, 2001).

6.14.4 Culture of single nematodes or pairs in hanging drops

To study the basic life-history traits (LHT) and reproductive biology of helminthic–bacterium complexes, a hanging drop method (Muschiol and Traunspurger, 2007) of a semi-solid nematode growth gelrite medium (NGG) is used. It allows the observation of single individuals of similar age with reliable accuracy, providing detailed information on, for example, nematode age at sexual maturity, lifespan, net reproductive rate, total fertility rate, generation time, intrinsic rate of natural increase, population doubling time and somatic growth rate. This method is described for *Steinernema riobrave* by Addis *et al.* (2014). Suspensions of the nematode-symbiotic bacteria are produced in nematode liquid medium, containing 15 g l^{-1} yeast extract, 20 g l^{-1} soy flour, 6 g l^{-1} lecithin + oil (1:1), 30 g l^{-1} vegetable oil, 4 g l^{-1} NaCl, 0.35 g l^{-1} KCl, 0.3 g l^{-1} $CaCl_2$, 0.2 g l^{-1} $MgSO_4 \times 7\ H_2O$, adjusted to pH 6.7. Cultures are incubated for 48 h and the suspension centrifuged at moderate speed for 10 min at 4°C. The supernatant is discarded and cells are washed with

sterilized K-medium (containing 3.1 g l^{-1} NaCl and 2.4 g l^{-1} KCl) and again centrifuged. The pellet is then dissolved in semi-solid NGG to obtain bacterial densities of, for example, 5 ×, 10 × and 20 × 10^9 bacterial cells ml^{-1}. Semi-solid NGG medium contains 1 g l^{-1} peptone from casein, 3 g l^{-1} NaCl and 1.5 g l^{-1} gellan gum (Gelrite; SERVA, Heidelberg, Germany). To 1 l of this medium, 1 ml of a 14.7 g l^{-1} suspension of $CaCl_2 \times 2\ H_2O$, 1 ml of 24.66 g l^{-1} $MgSO_4 \times 7\ H_2O$ and 25 ml of 13.6 g l^{-1} KH_2PO_4 buffer is added. Finally, 1 ml of 1 g l^{-1} cholesterol suspended in ethanol (>99%) is added. Ten µl of bacteria suspension in semi-solid NGG mixture drops is then pipetted on the inner side of lids of multi-well plates with 12 wells of 2.2 cm diam. The wells are filled with moist cellulose tissue paper. Single or pairs of nematodes are transferred into these drops and incubated at 25°C for 24 h. In order to supply bacteria *ad libitum*, the nematodes are transferred to fresh drops every day. Monoxenic cultures to produce nematode inoculum for the hanging drops can be produced on solid NGG. For this medium the peptone concentration is increased to 2.5 g l^{-1} and Gelrite to 3 g l^{-1}.

The method has also been applied for *Heterorhabditis bacteriophora* (Addis *et al.*, 2016a), *S. feltiae* and *S. yirgalemense* (Addis *et al.*, 2016b, c). Using the bacterium *E. coli*, LHT of the nematodes *C. elegans* (Muschiol *et al.*, 2009), *Pristionchus pacificus* (Gilarte *et al.*, 2015) and *Panagrolaimus* spp. (Ayub *et al.*, 2014) have been published. The latter study included feeding with a eukaryotic, heterotrophic dinoflagellate (*Crypthecodinium cohnii*).

6.15 Cryopreservation of Nematodes

Cryopreservation of cells, tissues or whole organisms by cooling to sub-zero temperatures (typically –80°C or –196°C) is a suitable method for long-term preservation of nematodes and stock cultures; furthermore some genetic studies require maintenance of the original nematode population. Nematodes where cryopreservation has been successful include free-living nematodes, e.g. *Panagrellus redivivus*, *Turbatrix aceti* and *C. briggsae* (Hwang, 1970; Haight *et al.*, 1975), animal-parasitic nematodes *Haemonchus* spp., *Trichostrongylus colubriformis*, *Ostertagia circumcincta* and *Cooperia punctata* (Gill and Redwin, 1995; Jensen *et al.*, 2000), entomopathogenic nematodes *Steinernema* and *Heterorhabditis* spp. (Popiel and Vasquez, 1991; Curran *et al.*, 1992) and plant-parasitic nematodes *Aphelenchoides sacchari* (Hwang, 1970), *Bursaphelenchus* spp. (Riga and Webster, 1991; Irdani *et al.*, 2006), *D. dipsaci* (Sayre and Hwang, 1975), *Heterodera* spp. (Triantaphyllou and McCabe, 1989), *Meloidogyne* spp. (Bridge and Ham, 1985; Triantaphyllou and McCabe, 1989; Vanderbeek *et al.*, 1996) and *Pratylenchus thornei* (Galway and Curran, 1995). Elsen *et al.* (2007) described a cryopreservation protocol for *R. similis* using vitrification solution-based methods based on a mixture of cryoprotectants in combination with rapid cooling and thawing rates. The following protocol is by J. Rowe (Rothamsted Research, UK, 2010, personal communication).

6.15.1 Protocol for cryopreservation and revival of nematodes

First incubation:

- Settle the nematodes in 1 ml of tap water (better to have large numbers) at room temperature – do not centrifuge.
- After settling for *ca* 1 h (depends on size and type of nematode), replace the water with 1 ml 10% ethanediol and put the tubes on ice. Put the 70% solution of ethanediol on ice to cool.
- Keep the centrifuge tube containing nematodes in 10% ethanediol on ice for 1 h.

Second incubation:

- Add 1 ml of 70% ethanediol to the 10% solution in the tube containing nematodes. Incubate on ice for 3 h.
- After 3 h incubation on ice, remove as much liquid as possible but keep tube on ice.
- Cool the aluminium block on ice, but keep it dry.
- Have the pre-cut strips of filter paper ready (pre-cut to fit the cryotubes).

Freezing:

- Use an open polystyrene box standing inside a deep plastic tray to contain liquid nitrogen (LN_2), remembering to wear cryogenic gloves and eye protection when handling the LN_2.
- With a micropipette, load enough of the nematode suspension in ethanediol (e.g. 0.5 µl) to saturate a strip of filter paper previously placed on the pre-cooled aluminium block.
- As quickly as possible, snap freeze the strip in LN_2 by submerging it; the nematodes will not be lost.
- Put up to ten (but no more) strips into each cryotube. The tube should be kept submerged in LN_2, for example by insertion in a polystyrene strip with holes in it pinned to the base of the box containing the LN_2, or the LN_2 inside will boil away.
- Use the large forceps to handle the cryotubes. Screw up the tubes when full, using forceps. Put labelled tubes in a straw and store in LN_2.
- It is advisable to place specimens in duplicate tubes and to store these tubes in separate flasks. Enough nematodes should be placed on each strip to ensure revival of a reasonable number in good condition when they are required.

Revival:

- The revival temperature is of utmost importance. The optimum for many species appears to be 37°C. For each species revived, a beaker of tap water (100 ml) is kept in a water bath at 37°C.
- The cryotube is kept submerged in LN_2 in a wide-mouth flask while it is unscrewed. As quickly as possible, take the strips with small forceps and drop as many as are required into the appropriately labelled beaker at 37°C.
- Remove the beaker from the water bath. Leave for 30 min and then wash the paper strips in the water and remove them. With a microscope, it should now be possible to see the nematodes in the process of reviving.
- This method has given 90% revival of species of *Ditylenchus*, *Panagrellus*, *Aphelenchoides*, *Meloidogyne*, *Bursaphelenchus* and *Hexatylus*. The longest-lived cultures that have been successfully revived are cultures of *Meloidogyne* kept in LN_2 for 3 years. The method, however, is not good for *Pratylenchus*.

6.16 Acknowledgements

The first author thanks David Hooper for permission to use information from his chapter *Culturing nematodes and related experimental techniques* (Hooper, 1986). We also thank Liza Alejandra González Jiménez and Luis Pocasangre Enamorado (Earth University, Guácimo, Limón, Costa Rica) for the carrot disc protocol and images, Jassmine Zorrilla (Laboratory of Tropical Crop Improvement, Katholieke Universiteit Leuven, Belgium) for *in vitro* banana plantlet images and Federico López-Moya (University of Alicante, Spain) for the *Arabidopsis thaliana* image.

6.17 References

Addis, T., Teshome, A., Strauch, O. and Ehlers, R.-U. (2014) Life history trait analysis of the entomopathogenic nematode *Steinernema riobrave*. *Nematology* 16, 929–936. DOI: 10.1163/15685411-00002819

Addis, T., Demissie, S., Strauch, O. and Ehlers, R.-U. (2016a) Influence of bacterial density and mating on the life history trait of *Heterorhabditis bacteriophora*. *Nematology* 18, 963–972. DOI: 10.1163/15685411-00003008

Addis, T., Mijuškovic, N., Strauch, O. and Ehlers, R.-U. (2016b) Life history traits, liquid culture production and storage temperatures of *Steinernema yirgalemense*. *Nematology* 18, 367–376. DOI: 10.1163/15685411-00002966

Addis, T., Teshomme, A., Strauch, O. and Ehlers, R.-U. (2016c) Life history trait analysis of the entomopathogenic nematode *Steinernema feltiae* provides the basis for prediction of dauer juvenile yields in monoxenic liquid culture. *Applied Microbiology and Biotechnology* 100, 4357–4366. DOI: 10.1007/s00253-015-7220-y

Akhurst, R.J. (1980) Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology* 121, 303–309. DOI: 10.1099/00221287-121-2-303

Amin, A.N.N., Hayashi, S. and Bartlem, D.G. (2014) Robust *in vitro* assay system for quantitative analysis of parasitic root-knot nematode infestation using *Lotus japonicus*. *Journal of Bioscience and Bioengineering* 118, 205–213. DOI: 10.1016/j.jbiosc.2014.01.006

Anderson, R.V. and Coleman, D.C. (1977) The use of glass microbeads in ecological experiments with bacteriophagic nematodes. *Journal of Nematology* 9, 319–322
Atamian, H.S., Roberts, P.A. and Kaloshian, I. (2012) High and low throughput screens with root-knot nematodes *Meloidogyne* spp. *Journal of Visualized Experiments* 61, e 3629. DOI: 10.3791/3629
Atkins, S.D., Manzanilla-López, R.H., Franco, J., Peteira, B. and Kerry, B.R. (2005) A molecular diagnostic method for detecting *Nacobbus* in soil and in potato tubers. *Nematology* 7, 193–202. DOI: 10.1163/1568541054879539
Ayub, F., Strauch, O., Seychelles, L. and Ehlers, R.-U. (2014) Influence of cell density of *Escherichia coli* and the dinoflagellate *Crypthecodinium cohnii* on life history traits of the nematode *Panagrolaimus* sp. strain NFS 24-5, a potential larval food for marine aquaculture. *Nematology* 16, 419–426. DOI: 10.1163/15685411-00002774
Barcala, M., García, A., Caber, J., Casson, S., Lindsey, K., Favery, K., García-Casado, G., Solano, R., Fenoll, C. and Escobar, C. (2010) Early transcriptomic events in microdissected *Arabidopsis* nematode-induced giant cells. *The Plant Journal* 61, 698–712. DOI: 10.1111/j.1365-313X.2009.04098.x
Barker, K.R. (1963) Parasitism of tobacco callus and Kentucky bluegrass by *Aphelenchus avenae*. *Phytopathology* 53, 870. [Abstr.].
Barker, K.R. and Darling, H.M. (1965) Reproduction of *Aphelenchus avenae* on plant tissues in culture. *Nematologica* 11, 162–166. DOI: 10.1163/187529265X00023
Bavaresco, L. and Walker, M.A. (1994) Techniques for successfully establishing *Xiphinema index* in dual culture with grape. *American Journal of Enology and Viticulture* 45, 273–277.
Bedding, R.A. (1984) Large scale production, storage and transport of the insect-parasitic nematodes *Neoaplectana* spp. and *Heterorhabditis* spp. *Annals of Applied Biology* 104, 117–120. DOI: 10.1111/j.1744-7348.1984.tb05593.x
Behmand, T., Ozturk, L. and Elekçioğlu, I.H. (2017) Mass culturing of stem and bulb nematodes (*Ditylenchus dipsaci*) for use in screening and impression training on carrot discs. *International Journal of Environment, Agriculture and Biotechnology* 2, 3148–3150. DOI: 10.22161/ijeab/2.6.48
Bingefors, S. and Bingefors, S. (1976) Rearing stem nematode inoculum for plant breeding purposes. *Swedish Journal of Agricultural Research* 6, 13–17.
Bird, D.W., Goodman, R.H. and Mai, W.F. (1968) Observations on the embryogenesis of *Trichodorus christiei* (Nematodea: Diphtherophoroidea). *Canadian Journal of Zoology* 46, 292–293. DOI: 10.1139/z68-045
Bleve-Zacheo, T. and Zacheo, G. (1983) Early stages of disease in fig roots induced by *Xiphinema index*. *Nematologia Mediterranea* 11, 175–187.
Bridge, J. and Ham, P.J. (1985) A technique for the cryopreservation of viable juveniles of *Meloidogyne graminicola. Nematologica* 31, 185–189. DOI: 10.1163/187529285X00247
Brown, A.M.V., Howe, D.K., Wasala, S.K., Peetz, A.B., Zasada, I.A. and Denver, D.R. (2015) Comparative genomics of a plant-parasitic nematode endosymbiont suggest a role in nutritional symbiosis. *Genome Biology Evolution* 7, 2727–2746. DOI: 10.1093/gbe/evv176
Brown, A.M.V., Wasala, S.K., Howe, D.K., Peetz, A.B., Zasada, I.A. and Denver, D.R. (2016) Genomic evidences for plant-parasitic nematodes as the earliest *Wolbachia* hosts. *Scientific Reports* 6, 34955. DOI: 10.1038/srep34955
Brown, A.M.V., Wasala, S.K., Howe, D.K., Peetz, A.B., Zasada, I.A. and Denver, D.R. (2018) Comparative genomics of *Wolbachia-Cardinium* dual endosymbiosis in a plant-parasitic nematode. *Frontiers in Microbiology* 9, 2482. DOI: 10.3389/fmicb.2018.02482
Brown, S.M. and Vessey, J.C. (1985) Rearing of *Radopholus similis* on banana fruit callus. *Revue de Nématologie* 8, 179–190.
Cabrera Poch, H.L., Manzanilla-López, R.H. and Kanyuka, K. (2006) Functionality of resistance gene *Hero*, which controls plant root-infecting potato cyst nematodes, in leaves of tomato. *Plant, Cell & Environment* 29, 1372–1378. DOI: 10.1111/j.1365-3040.2006.01517.x
Cabrera Poch, H.L., Manzanilla-López, R.H. and Clark, S.J. (2007) Ecotypes of the model legume *Lotus japonicus* vary in their interaction phenotypes with the root-knot nematode *Meloidogyne incognita*. *Annals of Botany* 99, 1223–1229. DOI: 10.1093/aob/mcm058
Cabrera, J., Díaz-Manzano, F.E., Sánchez, M. *et al*. (2014) A role for LATERAL ORGAN BOUNDARIES-DOMAIN 16 during the interaction *Arabidopsis-Meloidogyne* spp. provides a molecular link between lateral root and root-knot nematode feeding site development. *New Phytologist* 203, 632–645. DOI: 10.1111/nph.12826
Cabrera, J., Díaz-Manzano, F.E., Barcala, M., Arganda-Carreras, I., Almeida-Engler, J., Engler, G., Fenoll, C. and Escobar, C. (2015) Phenotyping nematode feeding sites: three-dimensional reconstruction and volumetric measurements of giant cells induced by root-knot nematodes in *Arabidopsis*. *New Phytologist* 206, 868–880. DOI: 10.1111/nph.13249
Cabrera, J., Barcala, M., García, A. *et al*. (2016) Differentially expressed small RNAs in Arabidopsis galls formed by *Meloidogyne javanica*: a functional role for miR390 and its TAS3-derived tasiRNAs. *New Phytologist* 209, 1625–1640. DOI: 10.1111/nph.13735

Castro, M.E.A. and Ferraz, S. (1990) Multiplication of *Pratylenchus brachyurus*, *P. zeae*, *Radopholus similis*, and *Tylenchorhynchus* sp. in monoxenic culture on alfalfa callus. *Nematologica Brasileira* 14, 103–120.
Chantanao, A. and Jensen, H.J. (1969) Saprozoic nematodes as carriers and disseminators of plant pathogenic bacteria. *Journal of Nematology* 1, 216–218.
Chapman, R.A. and Eason, J. (1974) Convenient storage of cysts of *Heterodera trifolii*. *Journal of Nematology* 6, 55–56.
Chen, T.-A. (1964) A technique for the selection and disinfestations of individual nematodes. *Phytopathology* 54, 127.
Chen, T.-A. and Rich, A.E. (1962) The role of *Pratylenchus penetrans* in the development of strawberry black root rot. *Plant Disease Reporter* 46, 839–843.
Chen, T.-A., Kierman, J. and Rich, A.E. (1961) Sterile culture techniques as tools in plant nematology research. *Phytopathology* 51, 799–800.
Chen, T.-A., Kierman, J. and Mai, W.F. (1965) A technique for studying the feeding of *Trichodorus christiei* on isolated root cells of corn. *Phytopathology* 55, 490–491.
Cohn, E. (1970) Observations on the feeding and symptomatology of *Xiphinema* and *Longidorus* on selected host roots. *Journal of Nematology* 2, 167–173.
Cordero, A.D. and Baldwin, J.G. (1990) Effect of age on body wall cuticle morphology of *Heterodera schachtii* Schmidt females. *Journal of Nematology* 22, 356–361.
Cordero, A.D., Baldwin, J.G. and Mundo-Ocampo, M. (1991) Fine structure of the posterior cone females of *Cactodera cacti* Filip'ev & Schuurmans Stekhoven (Nemata: Heteroderidae). *Revue de Nématologie* 14, 455–465.
Curran, J., Gilbert, C. and Butler, K. (1992) Routine cryopreservation of isolates of *Steinernema* and *Heterorhabditis* spp. *Journal of Nematology* 24, 269–270.
Cryan, W.S. (1963) A method for axenizing large numbers of nematodes. *Journal of Parasitology* 49, 351–352. DOI: 10.2307/3276014
De Ley, P. and Mundo-Ocampo, M. (2004) Cultivation of nematodes. In: Chen, Z.X., Chen, S.Y. and Dickson, D.W. (eds) *Nematology Advances and Perspectives. Volume 1. Nematode Morphology, Physiology and Ecology*. CAB International, Wallingford, UK, pp. 549–619.
Dallimore, C.E. (1966) Culture of *Ditylenchus destructor* on aseptic potato plugs. *Phytopathology* 56, 874–875. [Abstr.].
Darling, H.M., Faulkner, L.R. and Wallendal, P. (1957) Culturing the potato rot nematode. *Phytopathology* 47, 7.
Das, S. and Raski, D.J. (1968) Vector efficiency in *Xiphinema index* in the transmission of grapevine fanleaf virus. *Nematologica* 14, 55–62. DOI: 10.1163/187529268X00642
Den Ouden, H. (1960) An improved method for making thin agar layers in polyethylene bags. *Nematologica* 5, 255–259. DOI: 10.1163/187529260X00055
Denver, D.R., Brown, A.M.V., Howe, D.K., Peetz, A.B. and Zasada, I.A. (2016) Genome skimming: a rapid approach to gaining diverse biological insights into multicellular pathogens. *PLoS Pathogens* 12, e1005713. DOI: 10.1371/journal.ppat.1005713
Deubert, K.H., Norgren, R.L., Paracer, S.M. and Zuckerman, B.M. (1967) The influence of *Tylenchus agricola* and *Tylenchorhynchus claytoni* on corn roots under gnotobiotic conditions. *Nematologica* 13, 56–62. DOI: 10.1163/187529267X00913
Díaz-Manzano, F.E., Olmo, R. Cabrera, J., Barcala, M., Escobar, C. and Fenoll, C. (2016) Long-term *in vitro* system for maintenance and amplification of root-knot nematodes in *Cucumis sativus* roots. *Frontiers in Plant Science* 7, 124. DOI: 10.3389/fpls.2016.00124
Dolliver, J.S., Hildebrandt, A.C. and Riker, A.J. (1962) Studies of reproduction of *Aphelenchoides ritzemabosi* (Schwartz) on plant tissues in culture. *Nematologica* 7, 294–300. DOI: 10.1163/187529262X00585
Dougherty, E.C. (1959) Introduction to axenic cultures of invertebrate metazoan: a goal. *Annals of the New York Academy of Sciences* 77, 27–54. DOI: 10.1111/j.1749-6632.1959.tb36891.x
Dougherty, E.C., Hansen, E.L., Nicholas, W.L., Mollet, J.A. and Yarwood, E.A. (1959) Axenic cultivation of *Caenorhabditis briggsae* (Nematoda: Rhabditidae) with unsupplemented and supplemented chemically defined media. *Annals of the New York Academy of Sciences* 77, 176–217. DOI: 10.1111/j.1749-6632.1959.tb36901.x
Dropkin, V.H. and Boone, W.R. (1966) Analysis of host-parasite relationships of root-knot nematodes by single-larva inoculations of excised tomato roots. *Nematologica* 12, 225–236. DOI: 10.1163/187529266X00644
Dropkin, V.H. and Webb, R.E. (1967) Resistance of axenic tomato seedlings to *Meloidogyne incognita acrita* and to *M. hapla*. *Phytopathology* 57, 584–587.
DuCharme, E.P. and Hanks, R.W. (1961) Gnotobiotic techniques and the study of *Radopholus similis* on citrus. *Plant Disease Reporter* 45, 742–744.
Ehlers, R.-U. (2001) Mass production of entomopathogenic nematodes for plant protection. *Applied Microbiology and Biotechnology* 56, 623–633. DOI: 10.1007/s002530100711

Ehlers, R.-U. and Shapiro-Ilan, D.I. (2005) Mass production of entomopathogenic nematodes. In: Grewal, P.S., Ehlers, R.-U. and Shapiro-Ilan, D.I. (eds) *Nematodes as Biological Control Agents*. CAB International, Wallingford, UK, pp. 65–78.

Ehlers, R.-U., Lunau, S., Krasomil-Osterfeld, K. and Osterfeld, K.H. (1998) Liquid culture of the entomopathogenic nematode bacterium complex *Heterorhabditis megidis*/*Photorhabdus luminescens*. *BioControl* 43, 77–86. DOI: 10.1023/A:1009965922794

Eisenback, J.D. and Zunke, U. (1998) Extraction, culturing and microscopy. In: Sharma, S.B. (ed.) *The Cyst Nematodes*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 141–155.

Elhady, A., Shimaa, A., Hallmann, J. and Heuer, H. (2018) Rhizosphere microbiomes modulated by pre-crops assisted plants in defense against plant-parasitic nematodes. *Frontiers in Microbiology* 9, article 1133. DOI: 10.3389/fmicb.2018.01133

Elsen, A., Lens, K., Nguyet, D.T.M., Broos, S., Stoffelen, R. and De Waele, D. (2001) Aseptic culture systems of *Radopholus similis* for *in vitro* assays on *Musa* spp. and *Arabidopsis thaliana*. *Journal of Nematology* 33, 147–151.

Elsen, A., Valterra, S.F., Van Wauwe, T., Thuy, T.T.T., Swennen, R., De Waele, D. and Panis, B. (2007) Cryopreservation of *Radopholus similis*, a tropical plant-parasitic nematode. *Cryobiology* 55, 148–157. DOI: 10.1016/j.cryobiol.2007.06.006

Endo, B.Y. (1979) The ultrastructure and distribution of an intracellular bacterium-like microorganism in tissue of larvae of the soybean cyst nematode, *Heterodera glycines*. *Journal of Ultrastructural Research* 67, 1–14. DOI: 10.1016/s0022-5320(79)80012-x

Eriksson, K.B. (1980) Nematode inoculum propagated in monoxenic plant tissue cultures – nematode bank. *EPPO Bulletin* 10, 371–380. DOI: 10.1111/j.1365-2338.1980.tb02712.x

Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (eds) (1984) *Handbook of Plant and Cell Culture. Vol. I. Techniques for Propagation and Breeding*, Macmillan, New York, New York, USA.

Eyre, J.M. and Caswell, E.P. (1991) Sterile culture of *Rotylenchulus reniformis* on tomato root with Gellan Gum as a supporting medium. *Journal of Nematology* 23, 229–231.

Faulkner, L.R. and Darling, H.M. (1961) Pathological histology, hosts, and culture of potato rot nematode. *Phytopathology* 51, 778–786.

Faulkner, L.R., Bower, D.B., Evans, D.W. and Elgin, J.H. (1974) Mass culturing of *Ditylenchus dipsaci* to yield large quantities of inoculum. *Journal of Nematology* 6, 126–129.

Feder, W.A. (1958) Aseptic culture of the burrowing nematode, *Radopholus similis* (Cobb) Thorne on excised okra roots tissues. *Phytopathology* 48, 392–393 [Abstr.].

Feldmesser, J. (1967) An *in vitro* technique for nematode inoculation and culture of plants. *Nematologica* 13, 141–142 [Abstr.].

Fenwick, D.W. (1956) The production of sterile viable larvae of the potato root eelworm, *Heterodera rostochiensis*. *Nematologica* 1, 331–336. DOI: 10.1163/187529256X00348

Galway, N.J. and Curran, J. (1995) Cryopreservation of *Pratylenchus* spp. *Journal of Nematology* 27, 483–485.

Gamborg, O.L., Miller, R.A. and Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50, 151–158. DOI: 10.1016/0014-4827(68)90403-5

Gilarte, P., Kreuzinger-Janik, B., Majdi, N. and Traunspurger, W. (2015) Life-history traits of the model organism *Pristionchus pacificus* recorded using the hanging drop method: comparison with *Caenorhabditis elegans*. *PLoS One* 10, e0134105. DOI: 10.1371/journal.pone.0134105

Gill, J.H. and Redwin, J.M. (1995) Cryopreservation of the first-stage larvae of Trichostrongylid nematode parasites. *International Journal for Parasitology* 25, 1421–1426. DOI: 10.1016/0020-7519(95)00074-7

Goddijn, O.J.M., Lindsey, K., van der Lee, F., Klap, J.C. and Sijmons, P.C. (1993) Differential gene expression in nematode-induced feeding structures of transgenic plants harbouring promoter-gusA fusion constructs. *The Plant Journal* 4, 863–973. DOI: 10.1046/j.1365-313X.1993.04050863.x

Goodman, R.M. and Chen, T.-A. (1967) Two methods for surface disinfestation of *Trichodorus christiei*. *Phytopathology* 57, 1216–1220.

Grundler, F.M.W., Böckenhoff, A., Schmidt, K-P., Sobczak, M., Golinowski, W. and Wyss, U. (1994) *Arabidopsis thaliana* and *Heterodera schachtii*: a versatile model to characterize the interaction between host plants and cyst nematodes. In: Lamberti, F., De Giorgi, C. and Bird, D. McK. (eds) *Advances in Molecular Plant Nematology*. Plenum Press, New York, New York, USA, pp. 171–180.

Haegeman, A., Vanholme, B., Jacob, J., Vandekerckhove, T.T.M., Claeys, M., Borgonie, G. and Gheysen, G. (2009) An endosymbiotic bacterium in a plant-parasitic nematode: member of a new *Wolbachia* supergroup. *International Journal for Parasitology* 39, 1045–1054. DOI: 10.1016/j.ijpara.2009.01.006

Hahn, N.J. (1967) A sterile culture chamber for plants. *Canadian Journal of Botany* 45, 283–286.

Haight, M., Frim, J., Pasternak, J. and Frey, H. (1975) Freeze-thaw survival of free-living nematode *Caenorhabdithis briggsae*. *Criobiology* 12, 479–505.

Hajihassani, A., Tenuta, M. and Gulden, R.H. (2017) Monoxenic rearing of *Ditylenchus weischeri* and *D. dipsaci* and microplot examination of the host suitability of yellow pea to *D. weischeri*. *Plant Protection Science* 53, 254–264. DOI: 10.17221/158/2016-PPS

Hashmi, G., Huettel, R.N., Hammerschlag, F.A. and Krusberg, L.R. (1994) Optimal levels of *Meloidogyne incognita* inoculum for infection of tomato and peach *in vitro*. *Journal of Nematology* 26, 531–534.

Hildebrandt, A.C., Riker, A.J. and Duggar, B.M. (1946) The influence of the composition of the medium on growth *in vitro* of excised tobacco and sunflower tissue cultures. *American Journal of Botany* 33, 591–597.

Hoagland, D.R. and Arnon, I. (1950) *The water-culture method for growing plants without soil*. Agricultural Experimental Station, College of Agriculture, University of California Berkeley, California, USA.

Högger, C. (1973) Preferred feeding site of *Trichodorus christiei* on tomato roots. *Journal of Nematology* 5, 228–229.

Hooper, D.J. (1986) Culturing nematodes and related experimental techniques. In: Southey, J.F. (ed.) *Laboratory Methods for Work with Plant and Soil Nematodes*. 6th edn. *Ministry of Agriculture, Fisheries and Food*. Reference Book 402. Her Majesty's Stationery Office, London, UK, pp. 133–157.

Howe, D.K., Smith, M., Tom, D.M., Brown, A.M.V., Peetz, A.B., Zadasa, I.A. and Denver, D.R. (2019) Analysis of nematode-endosymbiont coevolution in the *Xiphinema americanum* species complex using molecular markers of variable evolutionary rates. *Nematology* 21, 533–546. DOI: 10.1163/15685411-00003233.

Huang, G., Allen, R., Davis, E.L., Baum, T.J. and Hussey, R.S. (2006) Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. *Proceedings of the National Academy of Sciences of the United States of America* 103, 14302–14306. DOI: 10.73/pnas.0604698103

Huang, X. and Becker, J.O. (1997) *In vitro* culture and feeding behaviour of *Belonolaimus longicaudatus* on excised *Zea mays* roots. *Journal of Nematology* 29, 411–415.

Huang, X. and Becker, J.O. (1999) Life cycle and mating behaviour of *Belonolaimus longicaudatus* in gnotobiotic culture. *Journal of Nematology* 31, 70–74.

Huettel, R.N. (1985) Carrot disc culture. In: Zuckerman, B.M., Mai, W.F. and Harrison, M.B. (eds) *Laboratory Manual for Plant Nematology.* University of Massachusetts Agricultural Experimental Station, Amherst, Massachusetts, USA, pp. 153–154.

Huettel, R.N. (1989) *In vitro* screening of soybean cultivars to *Radopholus similis* and *R. citrophilus*. *Nematropica* 19, 143–149.

Huettel, R.N. (1990) Monoxenic culturing of plant-parasitic nematodes using carrot discs, callus tissues, and root explants. In: Zuckerman, B.M., Mai, W.F. and Krusberg, L.R. (eds) *Plant Laboratory Manual*. Revised edn. University of Massachusetts Agricultural Experimental Station, Amherst, Massachusetts, USA, pp. 163–172.

Huettel, R.N. and Hammerschlag, F.A. (1986) Influence of cytokinin on *in vitro* screening of peaches for resistance to nematodes. *Plant Disease* 70, 1141–1144. DOI: 10.1094/PD-70-1141

Huettel, R.N. and Rebois, R.V. (1985) Culturing plant parasitic nematodes. In: Zuckerman, B., Mai, W.F. and Harrison, M.B. (eds) *Laboratory Manual for Plant Nematology.* University of Massachusetts Agricultural Experimental Station, Amherst, Massachusetts, USA, pp. 155–158.

Hutangura, P., Jones, M.G.K. and Heinrich, T. (1998) Optimisation of culture condition for *in vitro* infection of tomato with the root-knot nematode *Meloidogyne javanica*. *Australasian Plant Pathology* 27, 84–89. DOI: 10.1071/AP98010

Hwang, S.W. (1970) Freezing and storage of nematodes in liquid nitrogen. *Nematologica* 16, 305–308. DOI: 10.1163/187529270X00324

Inserra, R.N. and O'Bannon, J.H. (1975) Rearing migratory nematodes in citrus callus and roots produced from citrus leaves. *Journal of Nematology* 7, 261–263.

Irdani, T., Carletti, B., Ambrogioni, L. and Roversi, P.F. (2006) Rapid cooling and storage of plant nematodes at –140°C. *Cryobiology* 52, 319–322. DOI: 10.1016/j.cryobiol.2006.01.001

Jatala, P. Jensen, H.J. and Russell, S.A. (1974) *Pristionchus lheritieri* as a carrier of Rhizobium japonicum. *Journal of Nematology* 6, 130–131.

Jenkins, W.R. (1964) A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Disease Reporter* 48, 692.

Jensen, J.R., Merlo, A. and Vieira-Bressan, M.C.R. (2000) Cryopreservation of first and infective stage larvae an the infectivity test by anigical inoculation of the nematode *Haemonchus placei* and *Cooperia punctata*. *Revista Brasileira de Parasitologia Veterinaria* 9, 175–177.

Johnigk, S.-A. and Ehlers, R.-U. (1999) *Endotokia matricida* in hermaphrodites of *Heterorhabditis* spp. and the effect of the food supply. *Nematology* 1, 717–726. DOI: 10.1163/156854199508748

Johnson, R.N. and Viglierchio, D.R. (1969) Sugar beet nematode (*Heterodera schachtii*) reared on *Beta vulgaris* root explants. *Nematologica* 15, 129–143. DOI: 10.1163/187529269X00173

Kaplan, D.T. and Davis, E.L. (1990) Improved nematode extraction from carrot disk culture. *Journal of Nematology* 22, 399–406.

Karakaş, M. (2007) Life cycle and mating behavior of *Zygotylenchus guevarai* (Nematoda: Pratylenchidae) on excised *Petroselinum crispum* roots. *Pakistan Journal of Biological Sciences* 10, 4152–4155. DOI: 10.3923/pjbs.2007.4152.4155

Khera, S. and Zuckerman, B.M. (1963) *In vitro* studies of host-parasite relationships of some plant-parasitic nematodes. *Nematologica* 9, 1–6. DOI: 10.1163/187529263X00016

Khera, S., Bhatnagar, G.C., Tikyani, M.G. and Nandkumar, C. (1969) Culturing of *Telotylenchus indicus* Siddiqi, 1960 on alfalfa callus tissue. *Labdev Journal of Science and Technology* 7B, 330–331.

Klinkenberg, C.H. (1963) Observations on the feeding habits of *Rotylenchus uniformis*, *Pratylenchus crenatus*, *P. penetrans*, *Tylenchorhynchus dubius* and *Hemicycliophora similis*. *Nematologica* 9, 502–506. DOI: 10.1163/187529263X00566

Ko, M.P. and Van Gundy, S.D. (1988) An alternative gelling agent for culture and studies of nematodes, bacteria, fungi, and plant tissues. *Journal of Nematology* 20, 478–485.

Ko, M.P., Schmitt, D.P. and Sipes, B. (1996) Axenizing and culturing endomigratory plant-parasitic nematodes using Pluronic F127, including its effects on population dynamics of *Pratylenchus penetrans*. *Journal of Nematology* 28, 115–123.

Krusberg, L.R. (1961) Studies on the culturing and parasitism of plant-parasitic nematodes, in particular *Ditylenchus dipsaci* and *Aphelenchoides ritzemabosi* on alfalfa tissues. *Nematologica* 6, 181–200. DOI: 10.1163/187529261X00027

Krusberg, L.R. and Blickenstaff, M.L. (1964) Influence of plant growth regulating substances on reproduction of *Ditylenchus dipsaci*, *Pratylenchus penetrans* and *Pratylenchus zeae* on alfalfa tissue cultures. *Nematologica* 10, 145–150. DOI: 10.1163/187529264X00772

Krusberg, L.R. and Sardanelli, S.R. (1984) Technique for axenizing nematodes. *Journal of Nematology* 16, 348. [Abstr.].

Kumar, P., Kumar, V., Mohan, S. and Walia, R.K. (2017) A soil-less system for culturing rice root-knot nematode, *Meloidogyne graminicola*. *Nematology* 19, 749–750. DOI: 10.1163/15685411-00003076

Lauritis, J.A., Rebois, R.V. and Graney, L.S. (1982) Technique for gnotobiotic cultivation of *Heterodera glycines* Ichinohe on *Glycine max* (L.) Merr. *Journal of Nematology* 14, 422–424.

Loewenberg, J.R., Sullivan, T. and Schuster, M.L. (1960) Gall induction by *Meloidogyne incognita* by surface feeding and factors affecting the behaviour pattern of the second-stage larvae. *Phytopathology* 50, 322–323.

Lohar, D.P. and Bird, D.McK. (2003) *Lotus japonicus*: a new model to study root-parasitic nematodes. *Plant and Cell Physiology* 44, 1176–1184. DOI: 10.1093/pcp/pcg146

Lopez-Moya, F., Escudero, N., Zavala-Gonzalez, E.A., Esteve-Bruna, D., Blázquez, M.A., Alabadi, D. and Lopez-Llorca, L.V. (2017) Induction of auxin biosynthesis and WO5 repression mediate changes in root development in *Arabidopsis* exposed to chitosan. *Scientific Reports* 7, 1683. DOI: 10.1038/s41598-017-16874-5

Lownsbery, B.F., Huang, C.S. and Johnson, R.N. (1967) Tissue culture and maintenance of the root-lesion nematode, *Pratylenchus vulnus*. *Nematologica* 13, 390–394. DOI: 10.1163/187529267X00625

Lunau, S., Stoessel, S., Schmidt-Peisker, A.J. and Ehlers, R.-U. (1993) Establishment of monoxenic inocula for scaling up *in vitro* cultures of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabditis* spp. *Nematologica* 39, 385–399. DOI: 10.1163/187529293X00330

Manzanilla-López, R.H. (1997) *Studies on the characterisation and bionomics of* Nacobbus aberrans *(Thorne, 1935) Thorne & Allen, 1944 (Nematoda: Pratylenchidae)*. Ph.D. Thesis, Department of Agriculture, University of Reading, UK.

Manzanilla-López, R.H., Esteves, I., Powers, S.J. and Kerry, B.R. (2011) Nutritional effects of potato and break crops on abundance of *Pochonia chlamydosporia* and other fungal parasites of root-knot and potato cyst nematodes. *Annals of Applied Biology* 159, 118–129. DOI: 10.1111/j.1744-7348.2011.00479x

Marin, D.H., Barker, K.R., Kaplan, D.T., Sutton, T.B. and Opperman, C.H. (2000) Development and evaluation of a standard method for screening for resistance to *Radopholus similis* in bananas. *Plant Disease* 84, 689–693. DOI: 10.1094/PDIS.2000.84.6.689

McClure, M.A. and Viglierchio, D.R. (1966) The influence of host nutrition and intensity of infection on the sex ratio and development of *Meloidogyne incognita* in sterile agar cultures of excised cucumber roots. *Nematologica* 12, 248–258. DOI: 10.1163/187529266X00662

Mendy, B., Wang'ombe, M.W., Radakovic, Z.S., Holbein, J., Ilyas, M., Chopra, D., Holden, N., Zipfel, C., Grundler, F.M.W. and Siddique, S. (2017) *Arabidopsis* leucine-rich repeat receptor-like kinase NILR1 is required for induction of innate immunity to parasitic nematodes. *PLoS Pathogens* 13, e1006284. DOI: 10.1371/journal.ppat.1006284

Mercer, C.F. (1990) Development of nematodes *Meloidogyne hapla* Chitwood and *Heterodera trifolii* Goffart in white clover. *Nematropica* 36, 227–236.

Mercer, C.F. and Grant, J.L. (1993) The development of *Meloidogyne hapla* (Nematoda: Tylenchida) in resistant and susceptible *Trifolium semipilosum*. *Proceedings of the New Zealand Grasslands Association* 47, 267–271.
Mitkowski, N. and Abawi, G. (2002) Monoxenic maintenance and reproduction of root-knot nematode (*Meloidogyne hapla*) on multiple species-species *in vitro* root culture systems. *Plant Cell Reports* 21, 14–23. DOI: 10.1007/s00299-002-0468-6
Moens, T. and Vincx, M. (1998) On the cultivation of free-living marine and estuarine nematodes. *Helgoländer Meeresuntersuchungen* 52, 115–139. DOI: 10.1007/BF02908742
Moody, E.H., Lownsbery, B.F. and Ahmed, J.M. (1973) Culture of the root-lesion nematode *Pratylenchus vulnus* on carrot disks. *Journal of Nematology* 5, 225–226.
Moriarty, F. (1964) The monoxenic culture of beet eelworm (*Heterodera schachtii* Schm.) on excised roots of sugar beet (*Beta vulgaris* L.). *Parasitology* 54, 289–293. DOI: 10.1017/S0031182000067925
Mountain, W.B. (1955) A method of culturing plant-parasitic nematodes under sterile conditions. *Proceedings of the Helminthological Society of Washington* 22, 49–52.
Mountain, W.B. (1960) Theoretical considerations of plant-nematode relationships, In: Sasser, J. and Jenkins, W.R. (eds) *Nematology: Fundamentals and Recent Advances with Emphasis on Plant Parasitic and Soil Forms*. University of North Carolina Press, Chapel Hill, North Carolina, USA, pp. 419–421.
Müller, J. (1978) L'élevage monoxénique d'*Heterodera schachtii* sur crucifères et son application pour la sélection de plantes résistantes. *Revue de Nématologie* 1, 47–52.
Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* 15, 473–497. DOI: 10.1111/j.1399-3054.1962.tb08052.x
Muschiol, D. and Traunspurger, W. (2007) Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes, *Panagrolaimus* sp. and *Poikilolaimus* sp. from chemoautotrophic Movile Cave, Romania. *Nematology* 9, 271–284. DOI: 10.1163/156854107780739117
Muschiol, D., Schroeder, F. and Traunspurger, W. (2009) Life cycle and population growth rate of *Caenorhabditis elegans* studied by a new method. *BMC Ecology* 9, 14. DOI: 10.1186/1472-6785-9-14
Myers, R.F. (1967) Axenic cultivation of *Aphelenchoides sacchari* Hooper. *Proceedings of the Helminthological Society of Washington* 34, 252–255.
Myers, R.F., Buecher, E.J. and Hansen, E.L. (1971) Oligidic medium for axenic culture of *Aphelenchoides* sp. *Journal of Nematology* 3, 197–198.
Myers, R.J., Feder, W.A. and Hutchens, P.C. (1965) The rearing of *Radopholus similis* (Cobb) on grapefruit, okra, *and alfalfa root callus tissues. Proceedings of the Helminthological Society of Washington* 32, 94–95.
Niebel, A., Barthels, N., de Almeida-Engler, J., Karimi, M., Vercauteren, I., Van Montagu, M. and Gheysen, G. (1994) *Arabidopsis thaliana* as a model host plant to study molecular interaction with root-knot and cyst nematodes, In: Lamberti, F., De Giorgi, C., Bird, D. McK. (eds) *Advances in Molecular Plant Nematology*. Plenum Press, New York, New York, USA, pp. 161–170.
Nitsch, J.P. and Nitsch, C. (1969) Haploid plants from pollen grains. *Science* 163, 85–87. DOI: 10.1126/science.163.3862.85
Noel, G.R. and Atibalentja, N. (2006) '*Candidatus* Paenicardinium endonii', an endosymbiont of the plant-parasitic nematode *Heterodera glycines* (Nemata: Tylenchida), affiliated to the phylum *Bacteroidetes*. *International Journal of Systematic and Evolutionary Microbiology* 56, 1697–1702. DOI: 10.1099/ijs.0.64234-0
O'Bannon, J.H. and Taylor, A.L. (1968) Migratory endoparasitic nematodes reared on carrot discs. *Phytopathology* 58, 385.
Orion, D. and Bar-Eyal, M. (1995) Observations on the life cycle and behavior of *Helicotylenchus multicinctus* in monoxenic culture on *Arabidopsis thaliana*. *Nematropica* 25, 67–70.
Orion, D., Wergin, W.P. and Endo, B.Y. (1980) Inhibition of syncytia formation and root-knot nematode development on cultures of excised tomato roots. *Journal of Nematology* 12, 193–203.
Orion, D., Wergin, W.P. and Endo, B.Y. (1995) Root cortical cell spherical bodies associated with an induced resistance reaction in monoxenic culture of *Meloidogyne incognita*. *Journal of Nematology* 27, 320–327.
Ortuño, N., Franco, J., Ramos, J., Oros, R., Main, G. and Montecinos, R. (2005) *Desarrollo del manejo integrado del nematodo rosario de la papa Nacobbus aberrans en Bolivia. Documento de trabajo No. 26*. Fundación PROINPA-Proyecto PAPA ANDINA, Cochabamba, Bolivia, pp. 51–52.
Pajuelo, E. and Stougaard, J. (2005) *Lotus japonicus* as a model system. In: Márquez, A.J., Stougaard, J., Udvardi, M., Parmiske, M., Spaink, H., Saalbach, G., Webb, J. and Chiurazzi, M. (eds). Lotus japonicus *Handbook*. Springer, Dordrecht, The Netherlands, pp. 3–24.
Palomares-Rius, J.E., Archidona-Yuste, A., Cantalapiedra-Navarrete, C., Prieto, P. and Castillo, P. (2016) Molecular diversity of bacterial endosymbionts associated with dagger nematodes of the genus *Xiphinema* (Nematoda: Longidoridae) reveals a high degree of phylogenetic congruence with their host. *Molecular Ecology* 25, 6225–6247. DOI: 10.1111/mec.13904

Paracer, S.M. and Zuckerman, B.M. (1967) Monoxenic culturing of *Dolichodorus heterocephalus* on corn root callus. *Nematologica* 13, 478. DOI: 10.1163/187529267X00805
Parra, J.R.P. (2012) The evolution of artificial diets and their interactions in science and technology. In: Panizzi, A.R. and Parra, J.R.P. (eds) *Insect Bioecology and Nutrition for Integrated Pest Management.* CRC Press, Boca Raton, Florida, USA, pp. 51–92.
Paul, H.C., Zijlstra, C. Leeuwangh, J.E., Krens, F.A. and Huizing, H.J. (1987) Reproduction of the beet nematode *Heterodera schachtii* Schm. on transformed root cultures of *Beta vulgaris* L. *Plant Cell Reports* 6, 379–381. DOI: 10.1007/BF00269566
Peacock, F.C. (1959) The development of a technique for studying the host/parasite relationship of the root-knot nematode *Meloidogyne incognita* under controlled conditions. *Nematologica* 4, 43–55. DOI: 10.1163/187529259X00354
Peng, Y. and Moens, M. (1999) Effects of surface sterilisation and cold storage on *in vitro* behaviour of *Pratylenchus penetrans*. *Nematology* 1, 647–653. DOI: 10.1163/156854199508603
Piquerez, S.J.M., Harvey, S.E., Beynon, J.L. and Ntoukakis, V. (2014) Improving crop disease resistance: lessons from research on *Arabidopsis* and tomato. *Frontiers in Plant Science* 5,671. DOI: 10.3389/fpls.2014.00671
Plowright, R.A. and Akehurst, T.E. (1992) Monoxenic culture of the Ufra nematode *Ditylenchus angustus*. *Fundamental and Applied Nematology* 15, 327–330
Polychronopoulos, A.G. and Lownsbery, B.F. (1968) Effect of *Heterodera schachtii* on sugar beet seedlings under monoxenic conditions. *Nematologica* 14, 526–534. DOI: 10.1163/187529268X00219
Popiel, I. and Vasquez, E.M. (1991) Cryopreservation of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*. *Journal of Nematology* 23, 432–437.
Postma, W.J., Slootweg, E.J., Rehman, S. *et al*. (2012) The effector SPRYSEC-19 of *Globodera rostochiensis* suppresses CC-NB-LRR-mediated disease resistance in plants. *Plant Physiology* 160, 944–954. DOI: 10.1104/pp.112.200188
Prasad, S.K. and Webster, J.M. (1967) Effect of temperature on the rate of development of *Nacobbus serendipiticus* in excised tomato roots. *Nematologica* 13, 85–90. DOI: 10.1163/187529267X00968
Pupavkina, G.M. (1971) [A method of culturing *Ditylenchus destructor* on plant tissues.] *Byulleten Vsesoyuznogo Instituta Gel'mintologii im K.I. Skryabina* 6, 77–78.
Rao, U., Mauchline, T.H. and Davies, K.G. (2012) The 16S rRNA gene of *Pasteuria penetrans* provide an early diagnostic of infection of root-knot nematodes (*Meloidogyne* spp.). *Nematology* 14, 799–804. DOI: 10.1163/156854112X627318
Reise, R.W., Huettel, R.N. and Sayre, R.M. (1987) Carrot callus culture of endoparasitic nematodes. *Journal of Nematology* 19, 387–389.
Riedel, R.M. and Foster, J.G. (1970) Monoxenic culture of *Ditylenchus dipsaci* and *Pratylenchus penetrans* with modified Krusberg's and White's media. *Plant Disease Reporter* 54, 251–254.
Riedel, R.M., Foster, J.G. and Mai, W.F. (1973) A simplified medium for monoxenic culture of *Pratylenchus penetrans* and *Ditylenchus dipsaci*. *Journal of Nematology* 5, 71–72.
Riga, E. and Webster, J.M. (1991) Cryopreservation of the pinewood nematode, *Bursaphelenchus* spp. *Journal of Nematology* 23, 438–440.
Robinson, M.P., Atkinson, H.J. and Perry, R.N. (1988) The association and partial characterisation of a fluorescent hypersensitive response of potato roots to the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. *Revue de Nématologie* 11, 99–107.
Roman, J. and Hirschmann, H. (1969) Morphology and morphometrics of six species of *Pratylenchus*. *Journal of Nematology* 1, 363–386.
Rössner, J. (1971) Sterile culture of plant parasitic nematodes in a hermetically sealed glass container. *Nematologica* 17, 320–321. DOI: 10.1163/187529271X00189
Russell, C.C. and Perry, V.G. (1966) Parasitic habit of *Trichodorus christiei* on wheat. *Phytopathology* 60, 917–918.
Sanwal, K.C. (1959) A simple method for rearing pure populations of the foliar nematode, *Aphelenchoides ritzemabosi*, in the laboratory. *Canadian Journal of Zoology* 37, 707–711. DOI: 10.1139/z59-071
Sarah, J.L., Sabatini, C. and Boisseau, M. (1993) Differences in pathogenicity to banana (*Musa* sp., cv. Poyo) among isolates of *Radopholus similis* from different production areas of the world. *Nematropica* 23, 75–79.
Sayre, R.M. and Hwang, S. (1975) Freezing and storing *Ditylenchus dipsaci* in liquid nitrogen. *Journal of Nematology* 7, 199–202.
Sawhney, R. and Webster, J.M. (1975) The role of plant growth hormones in determining the resistance of tomato plants to the root-knot nematode, *Meloidogyne incognita*. *Nematologica* 21, 95–103. DOI: 10.1163/187529275X00392
Schroeder, P.H. and Jenkins, W.R. (1964) Reproduction of *Pratylenchus penetrans* on root tissues grown on three media. *Nematology* 9 (year 1963), 327–331. DOI; 10.1163/187529263X00836
Seinhorst, J.W. and Bels, P.J. (1951) *Ditylenchus destructor* Thorne 1945 in champignons. *Tijdschrift over Plantenziekten* 57, 167–169.

Shah, S.J., Anjam, M.S., Mendy, B., Anwer, M.A., Habash, S.S., Lozano-Torres, J.L., Grundler, F.M.W. and Siddique, S. (2017) Damage-associated responses of the host contribute to defence against cyst nematodes but not root-knot nematodes. *Journal of Experimental Botany* 68, 5949–5960. DOI: 10.1093/jxb/erx374
Shepherd, A.M., Clark, S.A. and Kempton, A. (1973) An intracellular micro-organism associated with tissues of *Heterodera* spp. *Nematologica* 19 31–34. DOI: 10.1163/187529273X00060
Shepperson, J.R. and Jordan, W.C. (1968) A technique for isolating and maintaining cultures of *Meloidogyne*. *Proceedings of the Helminthological Society of Washington* 35, 196–108.
Sijmons, P.C., Grundler, F.M.W., von Mende, N., Burrows, P.R. and Wyss, U. (1991) *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *The Plant Journal* 1, 245–254. DOI: 10.1111/j.1365-313X.1991.00245.x
Singh, N.D. and Norton, D.C. (1970) Variability in host-parasite relationships of *Heterodera trifolii*. *Phytopathology* 60, 1834–1837. DOI: 10.1094/Phyto-60-1834
Skoog, F. and Tsui, C. (1948) Chemical control of growth and bud formation in tobacco stem segment and callus cultured in vitro. *American Journal of Botany* 35, 782–787. DOI: 10.1002/j.1537-2197.1948.tb08148.x
Slatko, B.E., Taylor, M.J. and Foster, M.J. (2010) The *Wolbachia* endosymbiont as an anti-filarial nematode target. *Symbiosis* 51, 55–65. DOI: 10.1007/s13199-010-0067-1
Steele, A.E. (1972) Development of *Heterodera schachtii* on large rooted crop plants and the significance of root debris as a substratum for increasing field infestations. *Journal of Nematology* 4, 250–256.
Stiernagle, T. (2006) *Maintainance of* C. elegans. Available at: https://www.ncbi.nlm.nih.gov/books/NBK19649/ (accessed 21 July 2020).
Street, H.E. (ed.) (1973) *Plant Tissue and Cell Culture*. Botanical monographs, Vol. 11. Blackwell, Oxford, UK.
Sudirman and Webster, J.M. (1995) Effect of ammonium ions on egg hatching and second-stage juveniles of *Meloidogyne incognita* in axenic tomato root culture. *Journal of Nematology* 27, 346–352.
Sutherland, J.R. (1967) Parasitism of *Tylenchus emarginatus* on conifer seedling roots and some observations on the biology of the nematode. *Nematologica* 13, 191–196. DOI: 10.1163/187529267X00049
Sutherland, J.R. and Adams, R.E. (1965) The parasitism of red pine and other forest nursery crops by *Tylenchorhynchus claytoni* Steiner. *Nematologica* 10, 637–643. DOI: 10.1163/187529264X00286
Tamura, H. and Mamiya, Y. (1976) Reproduction of *Bursaphelenchus lignicolus* on alfalfa callus tissues. *Nematologica* 21 (year 1975), 449–454. DOI: 10.1163/187529275X00211
Tanda, A.S., Atwal, A.S. and Bajaj, Y.P. (1980) Reproduction and maintenance of root-knot nematode (*Meloidogyne incognita*) in excised roots and callus cultures of okra (*Abelmoschus esculentus*). *Indian Journal of Experimental Biology* 18, 1340–1342.
Tiner, J.D. (1960) Cultures of the plant parasitic nematode genus *Pratylenchus* on sterile excised roots. I. Their establishment and maintenance. *Experimental Parasitology* 9, 121–126. DOI: 10.1016/0014-4894(60)90019-9
Tiner, J.D. (1961) Cultures of the plant parasitic nematode genus *Pratylenchus* on sterile excised roots. II. A trap for collection of axenic nematodes and quantitative initiation of experiments. *Experimental Parasitology* 11, 231–240. DOI: 10.1016/0014-4894(61)90029-7
Tiner, J.D. (1966) Collection and storage of axenic inoculum of plant parasitic nematodes in the laboratory. *Annals of the New York Academy of Sciences* 139, 111–123. DOI: 10.1111/j.1749-6632.1966.tb41190.x
Townshend, J.L. (1974) Monoxenic culture of *Paratylenchus projectus*. *Nematologica* 20, 264–266. DOI: 10.1163/187529274X00267
Triantaphyllou, A.C. and McCabe, E. (1989) Efficient preservation of root-knot and cyst nematodes in liquid nitrogen. *Journal of Nematology* 21, 423–426.
Tyler, J. (1933) Reproduction without males in aseptic root cultures of the root-knot nematode. *Hilgardia* 7, 373–388. DOI: 10.3733/hilg.v07n10p373
Vanderbeek, H.J.G., Veldhuis, W.B.J., Zijlstra, C. and Vansilfhout, C.H. (1996) Preservation of *Meloidogyne hapla* and *M. chitwoodi* in liquid nitrogen: differences in response between populations. *Fundamental and Applied Nematology* 19, 227–234.
van der Walt, P.C.W. and De Waele, D. (1989) Mass culture of the potato nematode *Ditylenchus destructor* on groundnut tissue. *Phytophylactica* 21, 79–80.
Vanfleteren, J.R. (1978) Axenic culture of free-living, plant-parasitic, and insect-parasitic nematodes. *Annual Review of Phytopathology* 16, 131–157. DOI: 10.1146/annurev.py.16.090178.001023
Vanfleteren, J.R. (1980) Nematodes as nutritional models. In: Zuckerman, B.M. (ed.) *Nematodes as Biological Models*, Vol. 3. Academic Press, New York, New York, USA, pp. 47–79.
Verdejo, S., Jaffee, B.A. and Mankau, R. (1988) Reproduction of *Meloidogyne javanica* on plant roots genetically transformed by *Agrobacterium rhizogenes*. *Journal of Nematology* 20, 599–604.
Verdejo-Lucas, S. and Pinochet, J. (1992) Population densities of five migratory endoparasitic nematodes in carrot disks cultures. *Journal of Nematology* 24, 96–98.

Viglierchio, D.R. (1971) Race genesis in *Ditylenchus dipsaci*. *Nematologica* 17, 386–392. DOI: 10.1163/187529271X00611
Viglierchio, D.R. and Croll, N.A. (1968) Host resistance reflected in differential nematode population structures. *Science* 161, 271–272. DOI: 10.1126/science.161.3838.271-a
Viglierchio, D.R., Siddiqui, I.A. and Croll, N.A. (1973) Culturing and population studies of *Ditylenchus dipsaci* under monoxenic conditions. *Hilgardia* 42, 177–213. DOI: 10.3733/hilg.v42n06p177
Walsh, J.A., Lee, D.L. and Shepherd, A.M. (1983a) The distribution and effect of intracellular *Rickettsia*-like micro-organisms infecting adult males of the potato cyst-nematode *Globodera rostochiensis*. *Nematologica* 29 227–239. DOI: 10.1163/187529283X00474
Walsh, J.A., Shepherd, A.M. and Lee, D.L. (1983b) The distribution and effect of intracellular *Rickettsia*-like micro-organisms infecting second-stage juveniles of the potato cyst-nematode *Globodera rostochiensis*. *Journal of Zoology, London* 199 395–419. DOI: 10.1111/j.1469-7998.1983.tb02104.x
Wang, K.H., Sipes, B.S. and Kuehnle, A.R. (1997) Effect of soilless media on the growth of *Anthurium andraeanum* infected by *Radopholus similis*. *Nematropica* 27, 77–84.
Webster, J.M. (1966) Production of oat callus and its susceptibility to a plant parasitic nematode. *Nature* 212, 1472. DOI: 10.1038/2121472a0
Webster, J.M. and Lowe, D. (1966) The effect of the synthetic plant-growth substance, 2-4-dichlorophenoxyacetic acid, on the host-parasite relationship of some plant-parasitic nematodes in monoxenic callus culture. *Parasitology* 56, 313–322. DOI: 10.1017/s0031182000070918
Westcott, S.W. and Hussey, R.S. (1992) Feeding behaviour of *Criconemella xenoplax* in monoxenic culture. *Phytopathology* 82, 936–940. DOI: 10.1094/Phyto-82-936
White, P.R. (1943a) *A Handbook of Plant Tissue Culture*. The Jaques Cattell Press, Lancaster, Pennsylvania, USA.
White, P.R. (1943b) Nutrient deficiency studies and an improved inorganic nutrient for cultivation of excised tomato roots. *Growth* 7, 53–65.
White, P.R. (1963) *The Cultivation of Animal and Plant Cells*. 2nd edn, Ronal Press Co., New York, New York, USA.
Whitney, E.D. and Doney, D.L. (1970) Large scale hatching, disinfestation and storage of *Heterodera schachtii* larvae. *Phytopathology* 60, 1191–1194. DOI: 10.1094/Phyto-60-1191
Widdowson, E., Doncaster, C.C. and Fenwick, D.W. (1958) Observations on the development of *Heterodera rostochiensis* Woll. in sterile root cultures. *Nematologica* 3, 308–314. DOI: 10.1163/187529258X00076
Williams, T.D. (1963) The development of females of the potato root eelworm (*Heterodera rostochiensis* Woll.) on 'tuber pieces' of susceptible and resistant potatoes. *Euphytica* 12, 277–284. DOI: 10.1007/BF00027463
Winterhagen, P., Brendel, G., Krczal, G. and Reustle, G.M. (2007) Development of an *in vitro* dual culture system for grapevine and *Xiphinema index* as a tool for virus transmission. *South African Journal of Enology and Viticulture* 28, 1–5. DOI: 10.21548/28-1-1452
Wyss, U. (1970) Parasitierungsvorgang und Pathogenität wandernder Wurselnematoden an *Fragaria vesca semperflorens*. *Nematologica* 16, 55–62. DOI: 10.1163/187529270X00478
Wyss, U. (1971) Saugverhalten und Pathogenität von *Trichodorus* spp. in steriler Agarkultur. *Nematologica* 17, 501–517. DOI: 10.1163/187529271X00224
Wyss, U. (1978) Root and cell response to feeding by *Xiphinema index*. *Nematologica* 24, 159–166. DOI: 10.1163/187529278X00362
Yang, D., Chen, C., Liu, Q. and Jian, H. (2017) Comparative analysis of pre- and post-parasitic transcriptomes and mining pioneer effectors of *Heterodera avenae*. *Cell and Bioscience* 7, 11. DOI: 10.1186/s13578-017-0138-6
Zakrzewski, J. and Zakrewska, E. (1976) [*In vitro* breeding of red clover callus as a medium for propagating nematode – *Ditylenchus dipsaci* (Kühn).] *Hodowla Roslin Aklimatyzacja i Nasiennictwo* 20, 97–104.
Zuckerman, B.M. (1961) Parasitism and pathogenesis of the cultivated cranberry by some nematodes. *Nematologica* 6, 135–143. DOI: 10.1163/187529261X00397
Zuckerman, B.M. (1971) Gnotobiology. In: Zuckerman, B.M., Mai, W.F. and Rohde, R.A. (eds) *Plant Parasitic Nematodes Volume II.* Academic Press, New York, New York, USA, pp. 159–184.
Zuckerman, B.M. and Brzeski, M.W. (1966) Methods for the study of plant parasitic nematodes in gnotobiotic root culture. *Nematologica* 11, 453–466. DOI: 10.1163/187529265X00636

7 Measuring Nematodes and Preparation of Figures

Weimin Ye[1] and David J. Hunt[2]

[1]*Agronomic Division, North Carolina Department of Agriculture & Consumer Services, Raleigh, North Carolina, USA;* [2] *CABI, Egham, Surrey, UK*

7.1 Introduction

Despite the increasingly widespread use of molecular methodologies in diagnostics, accurate measurements remain an essential component for the description and identification of nematodes. Differences, albeit small yet consistent, can, if accurately recorded, be important for distinguishing taxa at the species level, although the usefulness and reliability of these characters may only be applicable to certain taxa. Measurement errors can be the result of the calibration of the optical system, operator accuracy or even by the same operator measuring the same specimen but on different occasions (see Frederick and Tarjan (1978) and Brown (1981), for example). Food source and other environmental factors often affect nematode dimensions and so the value/reliability of measurements, and hence the ratios calculated from these, have long been queried as to their appropriateness in taxon diagnostics, some values showing much more variability than others. Such natural variation occurs more so in some groups than others. For example, in entomoparasitic stages, where adult growth phase can be exceptional, most measurements may be far too variable to be of use, exceptions being those related to rigid cuticular structures such as the stylet or spicules, if present.

The way in which nematodes are prepared for study in temporary water mounts and the killing, fixing and processing methods employed also affect their morphometric characters, as does the way in which the slide mount is made (see Eisenback and Hunt, Chapter 5, this volume). For example, the cover slip needs to be supported by the use of such methods as paraffin wax or glass rods of appropriate diameter in order to avoid flattening the specimen and thus altering dimensions such as width (and therefore ratios based on that parameter) or distorting head or tail shape. It should also be borne in mind that older specimens have a tendency to flatten over time, maybe by a combination of natural processes, impact from an oil immersion lens or too much pressure being exerted during the cleaning of immersion oil from the cover slip.

All things considered, however, morphometrics, if taken accurately and used judiciously, can still be a useful supplement to identification as long as appropriate attention is paid to natural variation and practical knowledge of the systematic group involved. Sections of this chapter are modified and expanded from the excellent chapter by Hooper (1986).

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
DOI: 10.1079/9781786391759.0007

7.2 Calibration

Accurate calibration is the first step in measuring nematodes. Measurements can be accomplished using several different methods, choice of technique depending on what part of the nematode is being measured. Measuring using an eyepiece graticule or reticule, sketching the nematode part on paper by using a drawing tube (such devices have largely replaced the older *camera lucida*), or using measuring software on a computer screen image all require accurate calibration for each magnification employed. In order to calibrate the chosen method of measurement, a stage micrometer is used, which has an extremely accurate scale engraved or etched into the surface. The value of the scale is typically marked at one end of the micrometer, e.g. 1/0.01. This indicates that the entire scale is 1 mm long and that the smallest divisions are 0.01 mm (i.e. 10 µm) long. More expensive micrometers may be calibrated to 1.0 µm divisions. Having established the scale of the micrometer, place it on the microscope stage and focus using a lower powered objective. By rotating the eyepiece containing the eyepiece graticule and moving the stage, the graticule and micrometer scales can be made to line up and partially overlap. The points of coincidence between the eyepiece graticule scale and the stage micrometer scale (Fig. 7.1) can now be established. Read off the number of stage micrometer divisions equal to a known number of eyepiece graticule divisions. As the value of each division of the stage micrometer is known (see above), the value of an eyepiece graticule division can be easily calculated. For greater accuracy, as much of the stage micrometer scale length should be utilized as possible – this parameter will vary depending on objective magnification.

If, as in Fig. 7.1, we assume that the stage micrometer scale is 1 mm long with ten large divisions of 100 µm (smaller divisions may be 10 µm, or even 1 µm), and if, at the chosen magnification, ten large divisions of the eyepiece graticule extend, as depicted in Fig. 7.1, over 6.8 large divisions on the stage micrometer (10 × 6.8 = 680 µm), then each large division of the eyepiece graticule corresponds to 68 µm (i.e. 680 µm ÷ 10). When repeated for each objective, a series of calibration values will result. In the example cited above, and using a good optical setup, one would expect that the calibration value for a ×100 objective would be about one tenth of that for a ×10 objective, in this case around 6.8 µm. Note that the values are unlikely to be in absolutely direct proportion due to variations in the actual magnification of the objective lens, which may differ slightly from the nominal value engraved on the barrel.

If at any time an objective or eyepiece is changed, or an accessory such as a drawing tube is added or removed (adding a tube between the oculars and the objectives will increase focal length and hence magnification), the calibration **must** be repeated as the calibrated value is dependent upon the precise focal length/magnification of the optical system – an objective described as ×100 is unlikely to be precisely that power anyway and another example, even from the same manufacturer, will almost certainly differ slightly in its actual magnification due to production tolerances.

A drawing tube is calibrated slightly differently. In this case the stage micrometer, either entire or in part, depending on magnification, is drawn *via* the drawing tube at each magnification and the actual distance of

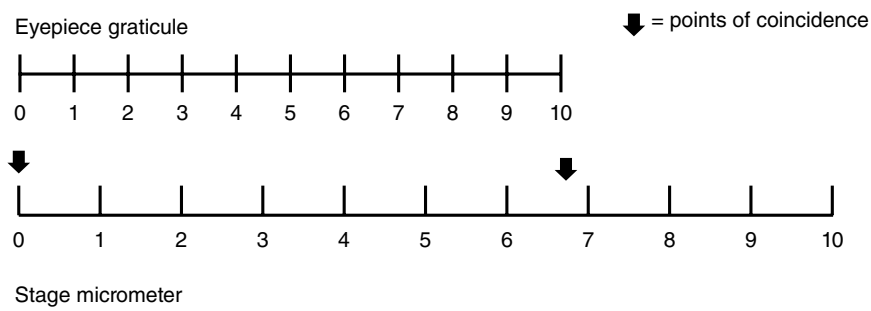

Fig. 7.1. Diagram showing calibration of an eyepiece graticule using a stage micrometer.

the drawn line calculated in microns for each magnification, this being done with reference to the calibration values of the stage micrometer. The line drawn for a given magnification can then be measured with a ruler in centimetres or millimetres and the final calibration done by dividing the value in microns by the measured value in cm or mm, the result being the number of microns per unit (cm or mm) measured. When a nematode structure is then drawn *via* the tube set to the same conditions, the length of that structure can be measured from the sketch in cm or mm and converted to microns using the previously calculated value appropriate to that magnification. Be aware that any adjustment to the magnification ratio of the drawing tube, be it by changing the ocular lens inside the tube, extending the length of the tube or increasing the distance between it and the drawing surface (by raising the microscope, for example) will change the calibration which must then be repeated for that particular set of parameters. Distances measured in this way may be converted to microns by simple multiplication, by extrapolation from a previously drawn calibration graph, by setting up a table of calculated values or *via* an Excel spreadsheet setup to do the calculations.

7.3 Direct Measurement

Some measurements, particularly those that are straight (e.g. stylet length or body width) can be made directly using a calibrated eyepiece graticule. A screw-micrometer eyepiece, similarly calibrated, can be used for more accurate measurements. Employing an eyepiece graticule to measure other values (e.g. body length, vulva position, tail length) depends on whether the nematode is straight, curved or coiled. If the nematode has died in a curved or coiled habitus, then the specimen should be either drawn *via* a drawing tube or measured on-screen using digital technology in order to get accurate values.

7.3.1 Computer measuring

Modern microscopes are usually equipped with a high-resolution digital camera capable of measuring nematodes and taking photos. The image from the microscope can be projected through a digital camera on to a computer monitor. Figure 7.2 is an example using the Zeiss Imager.A2 microscope and Zen 2 (Blue Edition) software to measure a female *Ditylenchus* species. A powerful measurement function using a cursor allows the user to measure directly the length, angle, circumference, diameter and area of the object in view. The computer calculates the required length, ratios, etc., and the data are displayed on a screen or provided as an Excel spreadsheet.

7.3.2 Drawing apparatus

The most common method of drawing nematodes involves the use of a drawing tube inserted between the microscope ocular head and the objective nosepiece. This device has a prism arrangement such that an image of the nematode can be projected on to a flat surface next to the microscope, the flat surface and specimen, under appropriately balanced lighting conditions, being simultaneously visible when looking down the microscope, as shown in Fig. 7.3. A changeable ocular lens within the body of the tube establishes the primary magnification factor, which may also be adjusted by a slider moving a lens system within the tube and/or by increasing the distance between the image forming surface of the tube and the drawing area. This technique has the advantage that the nematode structure can be traced effectively on a piece of paper fixed to the flat surface next to the microscope, both the nematode and the pencil/pen being visible when looking through the microscope binocular optics. The drawing tube is a development of the old *camera lucida*, which uses a semi-silvered prism and an angled mirror to project an image on to a surface. This latter device has a long pedigree but is seldom used today (see Hooper (1986) for details of the various setups).

Using a drawing tube effectively requires a balanced illumination between the microscope light source and the ambient light reflected into the tube from the drawing surface. This can be achieved by using, for example, an angle-poise lamp or other adjustable light source to reflect light off the drawing surface, the intensity being controlled by varying the distance between lamp and paper, and decreasing or increasing the illumination source of the microscope. When the illumination sources are appropriately balanced, the observer can

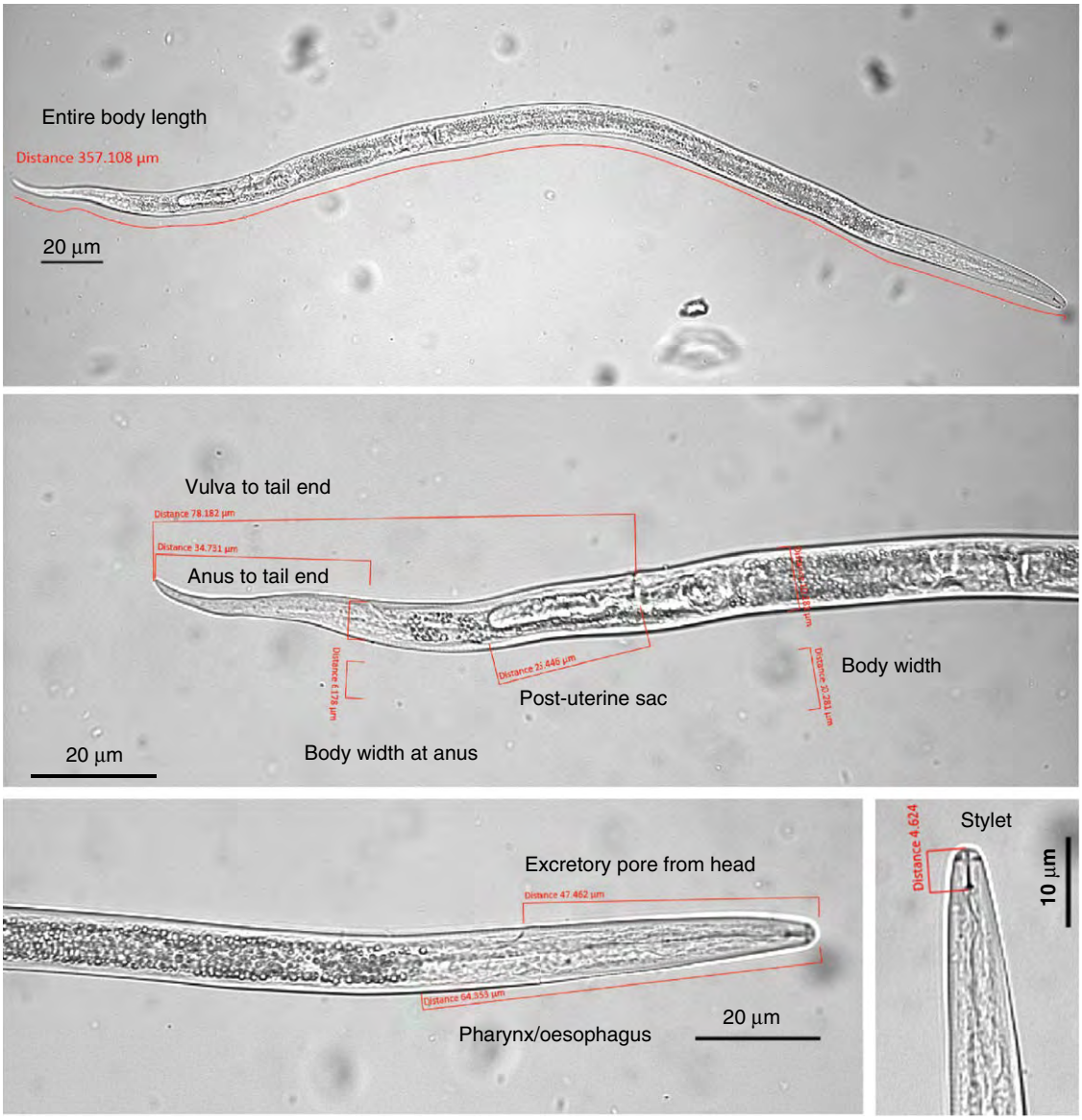

Fig. 7.2. Nematode measurement using Zeiss Imager.A2 microscope and Zen 2 (Blue Edition) software on a female *Ditylenchus* sp.

look down the microscope and see both the nematode and the drawing surface as a superimposed image. Occasionally, it may be necessary to boost the microscope illumination to discern detail of a particular structure before reducing the light to enable drawing to take place.

7.3.3 Drawing nematodes for measurement

Whether the objective is to draw freshly killed, fixed or processed and mounted specimens, an appropriate magnification should be chosen such that the nematode structures can be drawn either in a single section, or

Fig. 7.3. Compound microscope equipped with a drawing tube and light source for measurement and drawing.

when necessary and to preserve accuracy, in several delineated sections. The nematode can be drawn as a body outline but more often only the median line is depicted. The positions and extent of various larger organs can then be marked on this line, as shown in Fig. 7.4. Smaller dimensions, such as body width, stylet length, annulus width, etc., should be measured separately at a suitably higher magnification to preserve as much accuracy as possible for structures that may have a critical role to play in diagnostics. All of the drawings should have the magnification at which they were drawn written next to them so that the final calibration to µm is accurate.

Once the nematode and its parts have been drawn, the drawing can be measured, usually in mm. A rule is suitable for straight sections, but curved structures are best measured using a flexible but non-stretching material. In the past, lead fuse wire of suitable width was used but this is no longer available in many areas. Alternatives such as a thin non-stretchable rubber, plastic or string, lead-free solder wire, thick copper wire, nylon fishing wire, or a thin piece of single core electrical cable may be used by carefully bending to the shape of the structure and then straightening against a ruler to obtain the actual measurement in mm. Other methods include devices such as curvimeters or map measurers that can be run along the drawn line and then the value either calculated by running back along a ruler or read off directly. Such measurers need to be machined accurately and are more expensive but save time. Once the drawn specimen and its structures have been converted to mm, the previously calculated conversion factor(s) can be used to determine the actual length in µm.

7.3.4 Nematode measurement ruler

Most of the nematode measurements are not taken as straight lines but are curved; thus, accurate measurement cannot be achieved only by an eyepiece micrometer but by drawing the nematode outline for measurements *via* a drawing tube attached to the microscope. The latter method requires a ruler to convert the dimension and avoids the use of a calculator. To make this ruler, draw the scales of the stage

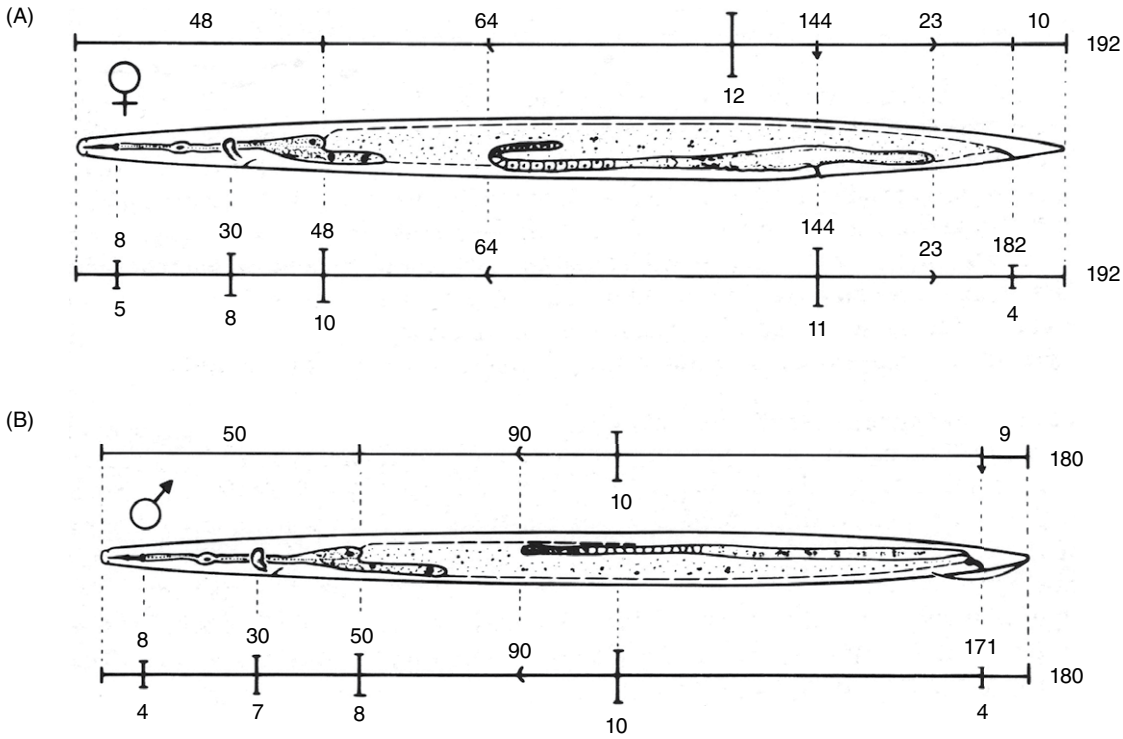

Fig. 7.4. Outline sketch of female **(A)** and male **(B)** nematode. The line drawn above each specimen (drawn through a *camera lucida* or drawing tube, for example at 240×) indicates the body proportions from which the de Man formula can be calculated; viz. Female: L = 192/240 = 0.8 mm; a = 192/12 = 16; b = 192/48 = 4; c = 192/10 = 19.2; V = 144/192 × 100 = *$^{3}75^{12}$**; *anterior ovary 64/192 × 100 = 33; **post-vulval sac 23/192 × 100 = 12; Male: L = 180/240 = 0.75 mm; a = 180/10 = 18; b = 180/50 = 3.6; c = 180/9 = 20; T = 90/180 × 100 = 50. The line drawn below each sketch indicates the distance of structures from the anterior end, with their relevant body widths, from which the Cobb formula is calculated. (Modified after Hooper, 1986.)

micrometer which is 1 mm ruled to 10 µm at each magnification on a sheet of paper through a drawing tube using 0.3 mm HB fine point pencil. Use Microsoft Office Word to design the ruler at each magnification by adjusting the font size and space of underlined symbols ↑ and | to match the stage micrometer. Print this paper sheet on a transparent sheet (Fig. 7.5) and place it on top of the outline sketch of the nematode if the line is straight, or with the aid of a flexible string or wire if the line is curved, to obtain the size.

7.3.5 Measurement criteria

De Man (1880) introduced a system in which certain body proportions were designated by the Greek letters α, β and γ, although these letters were subsequently replaced by a, b and c. Cobb (1890) devised a more complex formula for characterizing nematodes, although this, the Cobb formula, is no longer in common use. Many other morphometric criteria have been proposed, the majority of these being applied to certain groups of more specialized nematodes. The following morphometric criteria, although by no means a comprehensive listing, are in common use:

n = number of specimens.
L = total body length (mm or µm) as measured along the midline longitudinal axis.

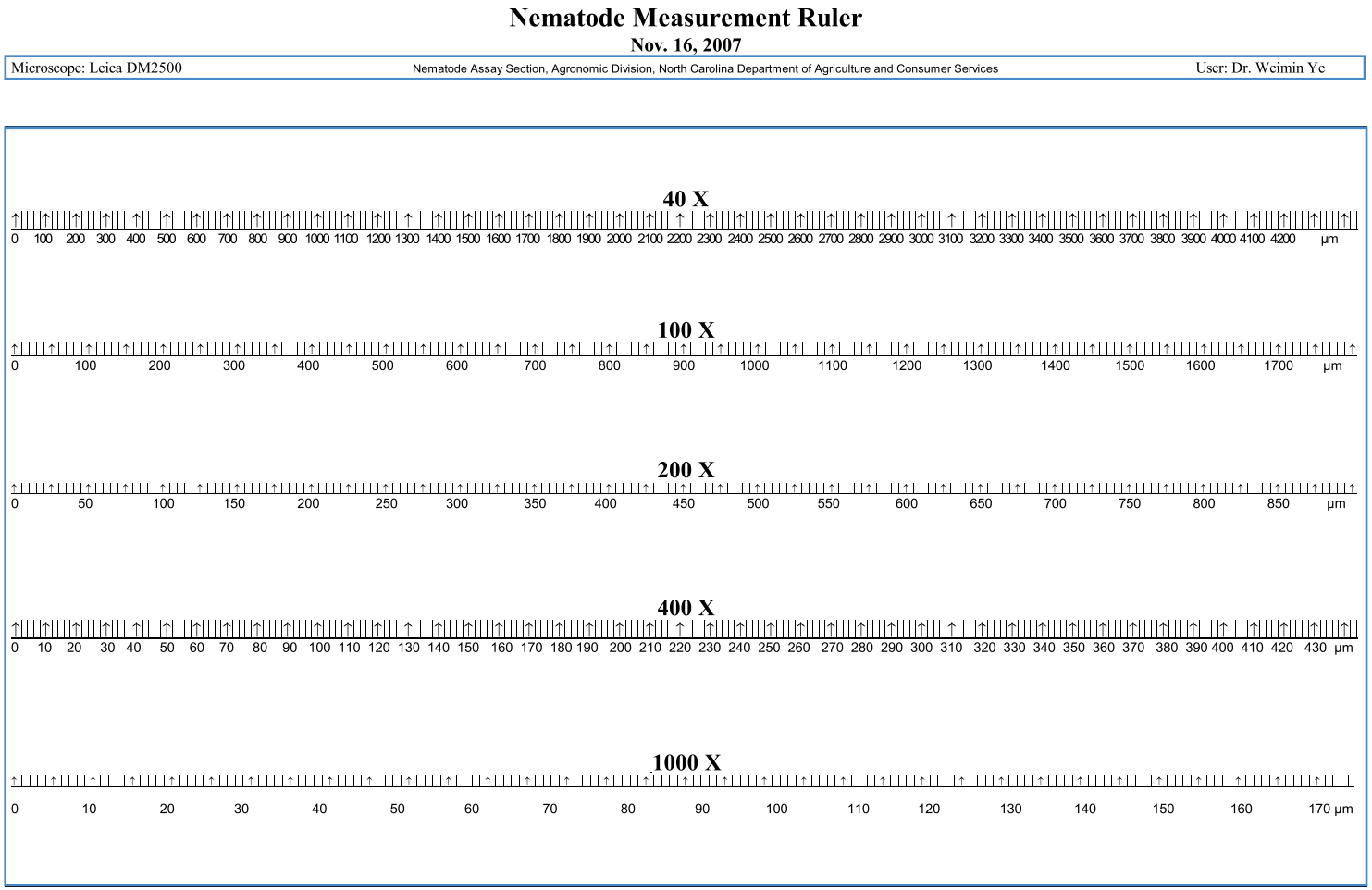

Fig. 7.5. Nematode measurement calibration ruler printed on a transparent sheet.

L' = body length from head to anus/cloacal aperture. Used when the tail is very long and often filamentous leading to breakage and underestimation of true length.
a = body length ÷ greatest body width/diameter, the latter being measured as a line intersecting the longitudinal body axis at a right angle.
b = body length ÷ distance from anterior end to pharyngo/oesophago-intestinal junction.
b' = body length ÷ distance from anterior end to posterior end of pharyngeal/oesophageal glands (used when glands overlap intestine).
c = body length ÷ tail length (as measured along the midline from anus or cloacal aperture to tail terminus).
c' = tail length ÷ body width at anus or cloacal aperture.
V = distance of vulva from anterior end × 100 ÷ body length. Small prefix and suffix numbers (e.g. V = $^{21}49^{20}$) indicate the proportions of body length from the vulva occupied by the anterior and posterior genital branches respectively, also expressed as percentages.
V' = Position of vulva from anterior end expressed as percentage of distance from head to anus. Used when the tail is very long and often filamentous leading to breakage and underestimation of true length and hence errors in V.
T = distance from cloacal aperture to anteriormost part of testis × 100 ÷ body length.

Length in µm of stoma or stylet (= 'stomatostyle' in Tylenchida; the dorylaimid stylet consists of an anterior 'odontostyle' plus a basal portion (extension or 'odontophore') which is measured separately).
Spicule length (usually measured along median line, the arc, but also as a straight line, the chord, between the distal and proximal ends) and gubernaculum length, in µm.
Other values sometimes used include:

a' = body length ÷ body width excluding cuticle, e.g. trichodorids (see Clark, 1963) or spines, e.g. *Criconema* spp. (see Mehta and Raski, 1971).
b_1 = body length ÷ distance from anterior end to the base of the median pharyngeal/oesophageal bulb.
G_1 = overall length of anterior genital branch (distance from vulva to terminal cell of ovary *via* flexure(s) when present) × 100 ÷ body length.
G_2 = overall length of posterior genital branch × 100 ÷ body length.
d = anterior end to guide ring ÷ body diameter at lip region (for longidorids; see Brown *et al*., 1994).
d' = body diameter at guide ring ÷ body diameter at lip region (for longidorids, see Brown *et al*., 1994).
DGO = distance from stylet base to orifice of dorsal gland opening (used in Tylenchida).
H or h = length of hyaline (clear) area in the tail between the body contents and the cuticle at the tail terminus.
J = length of hyaline tail region (for longidorids; see Lišková *et al*., 1997).
J' = hyaline region ÷ hyaline width (for longidorids; see Lišková *et al*., 1997).
K = width of stylet knobs ÷ height of stylet knobs (in Tylenchida: *Rotylenchus*, see Zancada *et al*., 1987).
M = length of anterior (conical) part of stylet (in Tylenchida) × 100 ÷ total stylet length.
O = distance from stylet base (in Tylenchoidea) to dorsal pharyngeal/oesophageal gland outlet × 100 ÷ total stylet length.
MB = Distance of median bulb from anterior end expressed as a percentage of total pharynx/oesophagus length.
P = distance of phasmid (when not erratic) from anus × 100 ÷ tail length (+ = anterior to anus, – = posterior to anus).
Pa = distance of anterior phasmid (when erratic) from anterior end × 100 ÷ body length.
Pp = distance of posterior phasmid (when erratic) from anterior × 100 ÷ body length.
rb = length of median bulb ÷ diameter of median bulb (in Tylenchida: *Rotylenchus*, see Zancada *et al*., 1987).
S = stylet length ÷ body width at base of stylet.
Caudal ratio A = length of hyaline tail divided by its proximal width.
Caudal ratio B = length of hyaline tail divided by its width at a point *5* µm from its terminus.

The ratios and measurements commonly used in cyst nematode descriptions are given below (see Subbotin *et al*., 2010):

a = body length ÷ greatest body width (male, second-stage juvenile (J2)) or L/W ratio (cyst, female, egg).

b = body length ÷ distance from anterior end to junction of pharynx and intestine (male, J2).
b' = body length ÷ distance from anterior end to posterior end of pharyngeal glands (male, J2).
c = body length ÷ tail length (male, J2).
c' = tail length ÷ body width at anus (male, J2).
DGO = distance from anterior end to orifice of dorsal gland opening (female, male, J2).
Granek's ratio = distance from the edge of the fenestra to the anus ÷ by the length of the fenestra (*Globodera* cyst vulval cone).
T = distance from cloacal aperture to anterior part of testis × 100 ÷ body length (male).

In the case of criconematid nematodes, additional metrics have been proposed based on annulus characteristics (see De Grisse, 1964):

R = number of body annuli.
RB = breadth of one body annulus in µm.
Rst= number of annuli between labial disc and base of stylet knobs.
ROes = number of annuli between labial disc and pharyngo/oesophago-intestinal valve.
Rhem = number of annuli between labial disc and first annulus posterior to the hemizonid.
Rex = number of annuli between labial disc and first annulus posterior to the excretory pore.
RV = number of annuli from tail terminus to vulva.
Ran = number of annuli from tail terminus to anus.
RVan = number of annuli between vulva and anus.

Loof and Coomans (published in 1970 but proposed in 1968) suggested a system, modified by Loof (1969), for locating pharyngeal/oesophageal gland nuclei in Dorylaimina. The positions are given as a percentage of the total pharynx/oesophagus length using the following abbreviations:

DN = position of the dorsal pharyngeal/oesophageal gland nucleus.
DO = position of the opening of the dorsal pharyngeal/oesophageal gland into the pharyngeal/oesophageal lumen.
S_1N_1; S_1N_2 = position of the anterior and posterior first subventral pair of pharyngeal/oesophageal nuclei, respectively.
S_1O_1; S_1O_2 = position of the anterior and posterior first subventral pair of pharyngeal/oesophageal gland openings, respectively.
S_2N_1, S_2N_2 = position of the anterior and posterior second subventral pair of pharyngeal/oesophageal nuclei, respectively.
S_2O_1, S_2O_2 = position of the anterior and posterior gland openings respectively; if opposite each other they are designated S_2N, S_2O.
K = distance DN to S_1N_1 as a percentage of the distance DN to S_1N_2.
K' = distance DO to S_1O_1, as a percentage of the distance DO to S_1O_2.

This system may be expressed as text or diagrammatically (see Fig. 7.6, but which is usually expressed with the lettering omitted) and shows the location of the expansion of the posterior pharyngeal/oesophageal bulb and the gland nuclei and their orifi. Anterior is to the left, where the sex of the nematode is also indicated, and the total length of the pharynx/oesophagus is cited at the right-hand end of the diagram. Loof and Coomans (1972) proposed a modification of the system specifically for longidorid nematodes, which have only three pharyngeal/oesophageal glands located in the expanded bulb, one dorsal and two ventrosublateral, instead of the usual five seen in the bulb of other dorylaims.

In entomopathogenic nematodes, such as *Steinernema* or *Heterorhabditis*, in addition to the standard de Man ratios, which often have little value except when measuring the non-feeding infective juvenile, the abbreviations listed below are usually employed (Nguyen and Hunt, 2007):

D% = labial region to excretory pore ÷ pharynx/oesophagus length × 100.
E% = labial region to excretory pore ÷ tail length × 100.

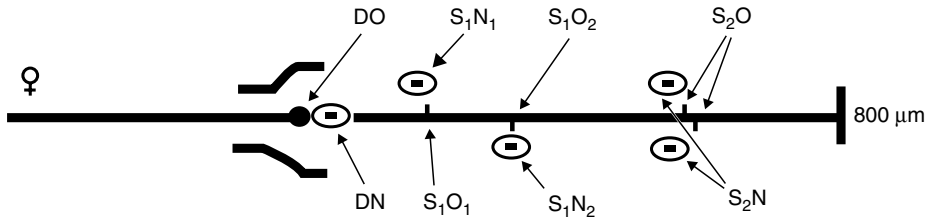

Fig. 7.6. Diagram showing the positions of the pharyngeal/oesophageal gland nuclei and their orifi in Dorylaimina following the system of Loof and Coomans (1968). Abbreviations: DN, position of the dorsal pharyngeal/oesophageal gland nucleus; DO, position of the orifi of the dorsal pharyngeal/oesophageal gland debouching into the pharyngeal/oesophageal lumen; S_1N_1; S_1N_2, position of the anterior and posterior first subventral pair of pharyngeal/oesophageal nuclei, respectively; S_1O_1; S_1O_2, position of the anterior and posterior first subventral pair of pharyngeal/oesophageal gland orifi, respectively; S_2N_1, S_2N_2, position of the anterior and posterior second subventral pair of pharyngeal/oesophageal nuclei, respectively; S_2O_1, S_2O_2, position of the anterior and posterior gland orifi respectively; if opposite each other they are designated S_2N, S_2O. (Redrawn after Hooper, 1986.)

SW% = spicule length ÷ body diameter at cloacal aperture × 100.
GS% = gubernaculum length ÷ spicule length × 100.
h% = length of hyaline region expressed as percentage of tail length.

Note that occasionally some of these values are cited as a simple ratio rather than being converted to a percentage, in which case they are referred to as D, E, SW and GS.

When measuring nematodes, it is advisable to map the position of each specimen on a permanent slide so that the measurements can be traced back to the original specimen, especially when missing data are subsequently needed. The number of specimens on a slide depends on the size of the nematodes, but the maximum number should usually not exceed ten. Figure 7.7 is an example of a sheet for recording the nematode specimen position for each microscope slide.

7.4 Processing Measurement Data and Preparing for Publication

Nematode measurements are usually presented in the format: mean ± standard deviation (range). About 20 specimens of each stage is usually an optimal number to represent a population, although this ideal may not be achievable due to limitations in the material available. Measuring more than 20 specimens from a single population is time consuming and usually relatively uninformative in terms of range and mean values, for example, and it is far more informative to measure additional individuals from different populations.

It is a lengthy process from collecting raw measurement data to final publication and errors may occur without a proper data handling procedure. Excel is the most commonly used software to accomplish such analyses. Ye (1996) proposed a simple method to develop a template when working with morphometric data so that the raw data are collected in a spreadsheet and the publication table is generated in the linked spreadsheet using embedded formulae. The characters should be preselected according to the nematode genus studied. Sheet 1 is the raw data table; sample information including nematode, locality, host and stage are keyed in the corresponding fields in bold. Formulae were predefined in shaded cells. Sheet 2 is linked to Sheet 1 to use as a Nematode Raw Measurement Collection Sheet, one specimen per sheet. This sheet has the same sequential order as Sheet 1 for convenient data entry and is used for the nematode outline sketch through a drawing tube or measurements obtained directly from the eyepiece graticule. Once the raw data is keyed into Sheet 1, the publication table (Sheets 3, 4) or text (Sheet 5) are automatically generated without further manipulation. This template can be modified to suit any nematode group and greatly assists in avoiding errors.

Sheet 1. Morphometrics of nematode species identification.

Nematode: ***Ibipora lolii*** Locality: **Uruguay**

Host: **Turfgrass** Stage: **Female**

(All measurements in µm)

No.:	1	2	3	4	5-15	16			n	Min	Max	AVG	SD
Slide No.:	4006	3879	3879	4005		3888							
Sample No.:	15-10619	15-10620	15-10620	15-10620		15-10625							
L	2560.0	2550.0	2940.0	2380.0		2820.0		L	16	2160.0	3100.0	2573.8	246.7
Anterior end to vulva	1300.0	1250.0	1480.0	1280.0		1460.0		Anterior end to vulva	16	1100.0	1600.0	1343.8	121.4
Pharynx	260.0	232.0	270.0	245.0		282.0		Pharynx	16	232.0	285.0	263.1	16.7
Mid-body width	40.0	44.0	50.0	40.0		50.0		Mid-body width	16	38.0	50.0	43.4	4.1
Tail	126.0	125.0	161.0	119.0		84.0		Tail	16	84.0	170.0	124.3	21.5
Anal body width	35.0	32.0	43.0	32.0		40.0		Anal body width	16	31.0	44.0	35.9	4.3
Excretory pore	200.0	206.0	242.0	220.0		225.0		Excretory pore	15	200.0	250.0	224.7	12.7
Stylet cone	75.0	67.0	80.0	83.0		80.0		Stylet cone	16	60.0	83.0	72.8	7.6
Stylet	108.0	104.0	103.0	118.0		117.0		Stylet	16	100.0	120.0	112.3	5.8
Lip height	9.0	7.0	10.0	11.0		10.0		Lip height	15	7.0	13.0	9.9	1.3
Lip width	20.0	17.0	16.0	16.0		19.0		Lip width	15	16.0	23.0	17.5	2.0
a	64.0	58.0	58.8	59.5		56.4		a	16	51.4	66.5	59.3	3.4
b	9.8	11.0	10.9	9.7		10.0		b	16	8.6	11.9	9.8	0.9
c	20.3	20.4	18.3	20.0		33.6		c	16	17.7	33.6	21.3	4.7
c'	3.6	3.9	3.7	3.7		2.1		c'	16	2.1	4.6	3.5	0.7
V	50.8	49.0	50.3	53.8		51.8		V	16	48.9	59.0	52.3	2.5

Sheet 2. Nematode raw measurement collection sheet.

Species:	*Ibipora lolii*		Comment:	No.:
Turfgrass	Uruguay	Slide No.:	Sample No.:	Female
L	Anterior end to vulva	Pharynx	Mid-body width	Tail
Anal body width	Excretory pore	Stylet cone	Stylet	Lip height
Lip width				
Description:				

Sheet 3. Morphometrics of *Ibipora lolii* from Uruguay (n = 16).

Character				Female
L	2573.8	±	246.7	(2160.0-3100.0)
a	59.3	±	3.4	(51.4-66.5)
b	9.8	±	0.9	(8.6-11.9)
c	21.3	±	4.7	(17.7-33.6)
c'	3.5	±	0.7	(2.1-4.6)
V	52.3	±	2.5	(48.9-59.0)
Anterior end to vulva	1343.8	±	121.4	(1100.0-1600.0)
Pharynx	263.1	±	16.7	(232.0-285.0)
Mid-body width	43.4	±	4.1	(38.0-50.0)
Tail	124.3	±	21.5	(84.0-170.0)
Anal body width	35.9	±	4.3	(31.0-44.0)
Excretory pore	224.7	±	12.7	(200.0-250.0)
Stylet cone	72.8	±	7.6	(60.0-83.0)
Stylet	112.3	±	5.8	(100.0-120.0)
Lip height	9.9	±	1.3	(7.0-13.0)
Lip width	17.5	±	2.0	(16.0-23.0)

Sheet 4. Morphometrics of *Ibipora lolii* from Uruguay.

Character	Female
n	16
L	2160.0-3100.0
	(2573.8±246.7)
a	51.4-66.5
	(59.3±3.4)
b	8.6-11.9
	(9.8±0.9)
c	17.7-33.6
	(21.3±4.7)
c'	2.1-4.6
	(3.5±0.7)
V	48.9-59.0
	(52.3±2.5)
Anterior end to vulva	1100.0-1600.0
	(1343.8±121.4)

Character	Female
Pharynx	232.0-285.0
	(263.1±16.7)
Mid-body width	38.0-50.0
	(43.4±4.1)
Tail	84.0-170.0
	(124.3±21.5)
Anal body width	31.0-44.0
	(35.9±4.3)
Excretory pore	200.0-250.0
	(224.7±12.7)
Stylet cone	60.0-83.0
	(72.8±7.6)
Stylet	100.0-120.0
	(112.3±5.8)
Lip height	7.0-13.0
	(9.9±1.3)
Lip width	16.0-23.0
	(17.5±2.0)

Sheet 5. Text format.

Morphometrics of *Ibipora lolii* from Uruguay (n = 16).
L = 2160.00 - 3100.00 (2573.75 ± 246.73) µm, a = 51.43 - 66.50 (59.33 ± 3.38), b = 8.58 - 11.92 (9.79 ± 0.85), c = 17.69 - 33.57 (21.34 ± 4.71), c' = 2.10 - 4.59 (3.51 ± 0.69), V = 48.87 - 59.02 (52.29 ± 2.49), VL = 1100.00 - 1600.00 (1343.75 ± 121.44) µm, Pharynx = 232.00 - 285.00 (263.06 ± 16.65) µm, Mid-body width = 38.00 - 50.00 (43.44 ± 4.06) µm, Tail = 84.00 - 170.00 (124.31 ± 21.49) µm, ABW = 31.00 - 44.00 (35.88 ± 4.27) µm, Excretory pore = 200.00 - 250.00 (224.67 ± 12.69) µm, Stylet cone = 60.00 - 83.00 (72.75 ± 7.62) µm, Stylet = 100.00 - 120.00 (112.31 ± 5.85) µm, Lip height = 7.00 - 13.00 (9.93 ± 1.29) µm, Lip width = 16.00 - 23.00 (17.47 ± 1.96) µm.

7.4.1 Cobb formula

Originally proposed in 1890 (Cobb, 1890), the Cobb formula consisted of 11 numbers representing 11 dimensions. This formula has nowadays almost entirely fallen out of favour, the emphasis in taxonomic descriptions shifting decisively to the de Man indices by the mid-20th century. The formula is often seen in older descriptions, however, and needs to be understood as it usually forms an integral part of the original description of older species. The Cobb formula usually consists of a horizontal line representing the nematode, head to the left, bearing five pairs of figures, each expressed as a percentage of the body length and a single terminal figure, the length in mm: quoting Cobb (1890) 'The unit of measurement is not absolute but relative…' and represents a hundredth of the length of the nematode. The upper number of each pair is the distance from the anterior end, the lower is the body width at that point. The first measurements (on the left) are taken at the base of the stoma or stylet, the second at the middle of the nerve ring, the third at the pharyngo/oesophageal intestinal junction, the fourth at either the vulva in females or at the middle (M) of the body in

Nematode Specimen Position Map

Nematode position	No	Nematode Species	Comment	J2	J3	J4	F	M	
Slide No.:	1								
	2								
	3								
	4								
	5								
	6								
	7								
	8								
	9								
	10								
Slide No.:	1								
	2								
	3								
	4								
	5								
	6								
	7								
	8								
	9								
	10								
Slide No.:	1								
	2								
	3								
	4								
	5								
	6								
	7								
	8								
	9								
	10								
Slide No.:	1								
	2								
	3								
	4								
	5								
	6								
	7								
	8								
	9								
	10								

Fig. 7.7. Nematode specimen position map.

males, and the fifth at the anus or cloacal aperture. The prefix and suffix attached to the fourth term indicate the extent of the ovaries expressed as a percentage of body length and additional hyphen or quotation marks were used to indicate the arrangement of the genital system and whether outstretched or reflexed (see Cobb, 1890). For the male testis/testes, a prefix is attached to the symbol 'M'. The male 'M' was also given additional qualifiers to indicate the number and direction of the genital tracts and whether outstretched or reflexed.

Thus, a nematode could have the following formulae:

♀	4.2	15.6	25	$^{33}75^{12}$	95	0.80 mm
	2.6	4.2	5.2	5.7	2.1	

♂	4.5	16.7	27.8	^{50}M	95	0.75 mm
	2.2	3.9	4.5	5.6	2.2	

Descriptions of new species should be as detailed as possible and contain all measurements likely to be of taxonomic value, although only a few measurements may be necessary for identification within a particular group.

7.4.2 Preparing figures for publication

7.4.2.1 Line drawings

Line drawings are usually an essential component in any paper describing nematodes and should be clear, accurately drawn and depict all major characters of the taxon. Although they may vary according to individual style, certain criteria should be satisfied. The drawings should be bold, clear and unambiguous, clearly showing the diagnostic features of the species concerned. Effects such as stippling or 3D shading are best avoided unless the preparer has artistic expertise in this area. Ideally, there should be at least one drawing of a complete nematode to show its general form, and preferably one of each sex. These drawings sometimes show only the outlines and positions of the main organs and structural features. It is often useful to show the typical heat-killed shape. Details of particular features, including those deemed to be diagnostic of the taxon, should be drawn at a higher magnification and enough figures should be presented to portray adequately any complex feature, taking care to note the orientation of the specimen (right, left, ventral, rolled, etc.) being depicted. The drawings should not be skimped, even if high-quality light micrographs and/or scanning electron micrographs are available, as it is important to give as complete an illustration as possible. Failure to do so may result in the taxon being regarded as *species inquirenda* at a later date. Accurate scales, not magnification indicators in the legend, should always be included in the figure.

Before the advent of digital methods, line drawings were usually done using Indian ink applied *via* a suite of variously sized stylograph pens on to high-quality artist's board or a suitable transparent or translucent film. Such drawings were usually done at two to three times the final linear reproduction size so that any errors or wobbles in line would be less obvious after reduction to fit the page size of the journal. Finished drawings were reproduced photographically using lithographic film, which only records black or white, to produce a suitably sized print for initial submission; the original drawings finally being packaged and sent to the publisher after acceptance. Hooper (1986) covers the older methodologies in detail which, although giving excellent results in skilled hands, are now rarely employed, being replaced by various manipulations in the digital domain. Nevertheless, the latter methods still require considerable knowledge and skill to execute to the required standard.

Standard drawings may still be made on suitable heavy-weight artist's card, but probably using a disposable stylograph-style or fine felt-tip pen, rather than the draughtsman's tools of yore. It is also possible to assemble a plate from individual pencil drawings, which can be digitally scanned as greyscale images, the curves then being adjusted in an image processing program such as Adobe Photoshop® to achieve the desired 'blackness' of line and whiteness of background canvas or left as greyscale toning. Drawings can also be made in proprietary vector graphics software such as Adobe Illustrator®, or freeware such as Inkscape®. In the digital domain, it is much easier to cut and paste or move individual drawings around in order to achieve the final figure, although care must be taken not to degrade image detail unduly as every manipulation has a cost. Line drawings may be saved as bitmaps (BMP), in which case the pixels only record black or white (there can be no greys), but they are often saved as greyscale, although the latter files

are very much larger. In the case of greyscale images, there can be problems with background 'noise', although this can usually be controlled by tweaking the image curves to boost the blacks of the lines and increase the whiteness of the background. Recalcitrant noise can then be removed with the eraser tool. Bitmap images should be saved as TIFF compressed using the LZW algorithm (a lossless format) at a minimum resolution of 600 dpi (*ca* 250 dots cm^{-1}) and preferably at 1000–1200 dpi (*ca* 400-475 dots cm^{-1}). File size remains small as only two parameters, black or white, are recorded in bitmaps. Greyscale images of line drawings should be saved as TIFF with LZW compression or maximum quality JPEG (known as a 'lossy' format as it increasingly throws data away with compression) at a minimum resolution of 600 dpi as 300 dpi (= *ca* 125 dots cm^{-1}) is usually insufficient for full-page line figures in a printed journal. Figures 7.8 and 7.9 are two good examples of line figures done either by hand (Fig. 7.8) or by computer (Fig. 7.9) when describing a new species.

7.4.2.2 Greyscale

Greyscale images are composed of 256 shades of grey. The files may be saved as popular formats such as TIFF or JPEG. JPEG files offer more compression than TIFF but, as already mentioned, lose data, although this is usually only critical when excessive compression is applied. Digital devices such as scanners, particularly if left to run on auto settings, often produce files of greyscale subjects in the RGB (red–green–blue colour channels) format. The unnecessary colour information in the RGB mode greatly inflates file size (note that a larger file size does not necessarily equate with better resolution) and should ideally be discarded using an image processing package before the file is saved.

7.4.2.3 Colour

If colour figures are required, they are usually saved as three channel RGB although some journals may stipulate CMYG (cyan–magenta–yellow–green). RGB files are usually 8-bits per channel (8 bpc), giving over 16 million possible colours. RBG files should be saved at a resolution (genuine, not interpolated upwards) of at least 300 dpi. The files may be saved in formats such as TIFF or JPEG as described above.

7.4.2.4 Combination artwork

Combination artwork involves a mixture of art types, such as line drawings with either greyscale or RGB colour images. Such files are saved either as greyscale or RGB colour, depending on content. Due to the line drawing element, resolution should be higher than that selected for straight greyscale or RGB images so as to minimize pixelation of non-vertical or non-horizontal lines – values of 500–600 dpi are usually adequate, but individual journals may stipulate a higher value. Save the combination image either as TIFF with LZW compression or as a maximum quality JPEG.

7.4.2.5 Labelling

Application of lettering and any annotations should be done after the figure has been finalised and only when it is at the desired final resolution. This avoids pixelation of the lettering, which occurs should letters be applied to a low-resolution canvas. Size of font should be chosen carefully so as to be clear but not obtrusive when reproduced, a smaller font being reserved for the values on scale bars or labelling of particular structures. Lettering may be in black or white, depending on background. Advanced image processing programs also have options that smooth the edges of applied lettering. The best fonts to use are either sans-serif fonts, such as Arial (or one of its variants), Helvetica or Times New Roman, the latter only if a serif font is preferred. Eschew fancier fonts (or Courier) as they often distract from the figure, looking particularly appalling in dendrograms, for example. Be aware that the journal may have specific font requirements for labelling.

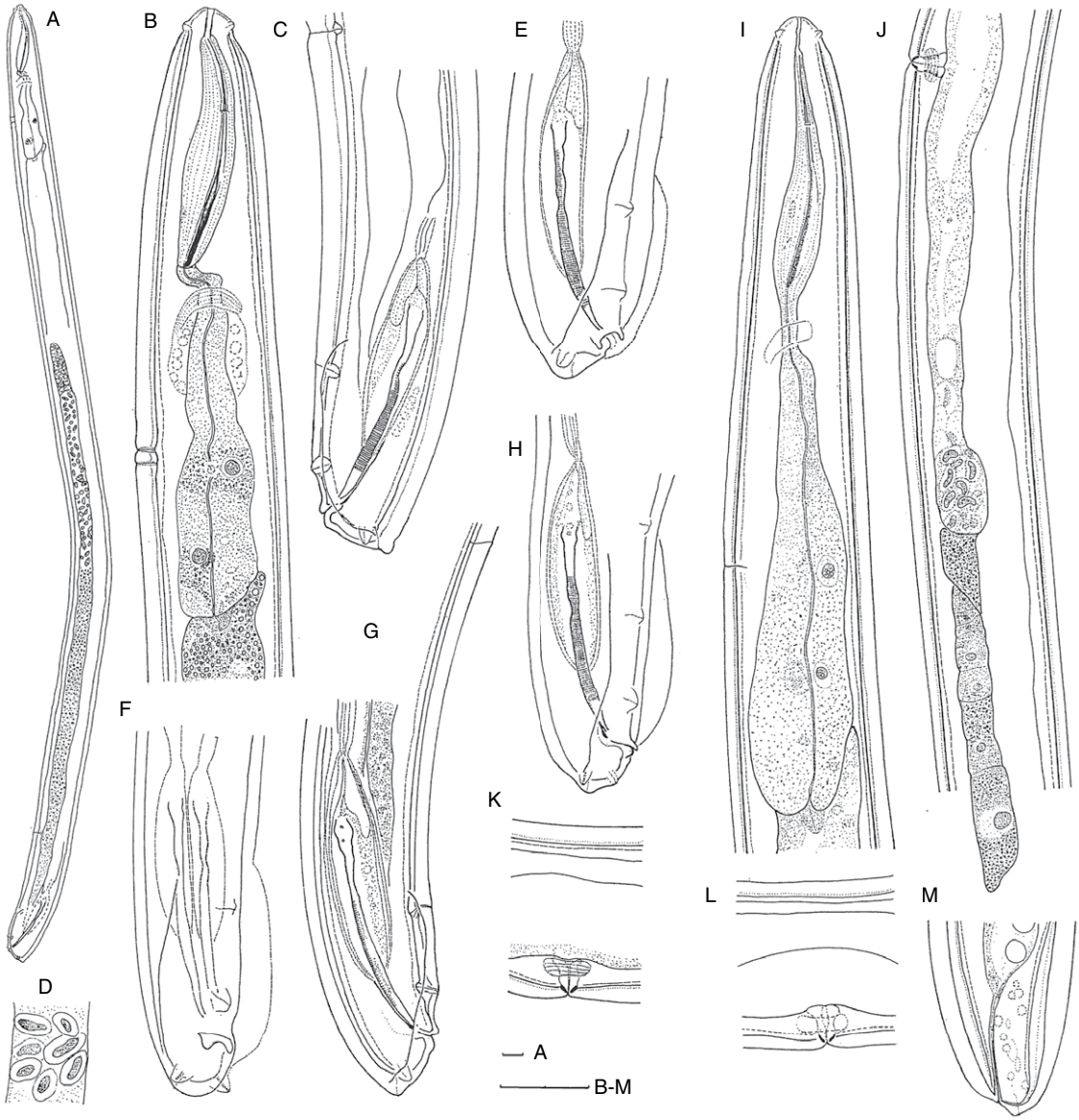

Fig. 7.8. An example of a nematode line drawing of *Paratrichodorus ramblensis* using a manual approach. (Reproduced from Decraemer *et al*., 2019.)

7.4.2.6 Encapsulated PostScript (EPS) files

EPS files are preferred by some journals for vector graphics. Resolution should be 1000 dpi for line art and 300 dpi for greyscale/RGB art. Be sure to check with the journal which file types are acceptable and the resolution requirements.

7.4.2.7 Layers

When composing a figure, the individual elements, including the lettering, are applied to the background or canvas. Typically, each component (and that includes all letters, arrows, etc., that are separately applied) will

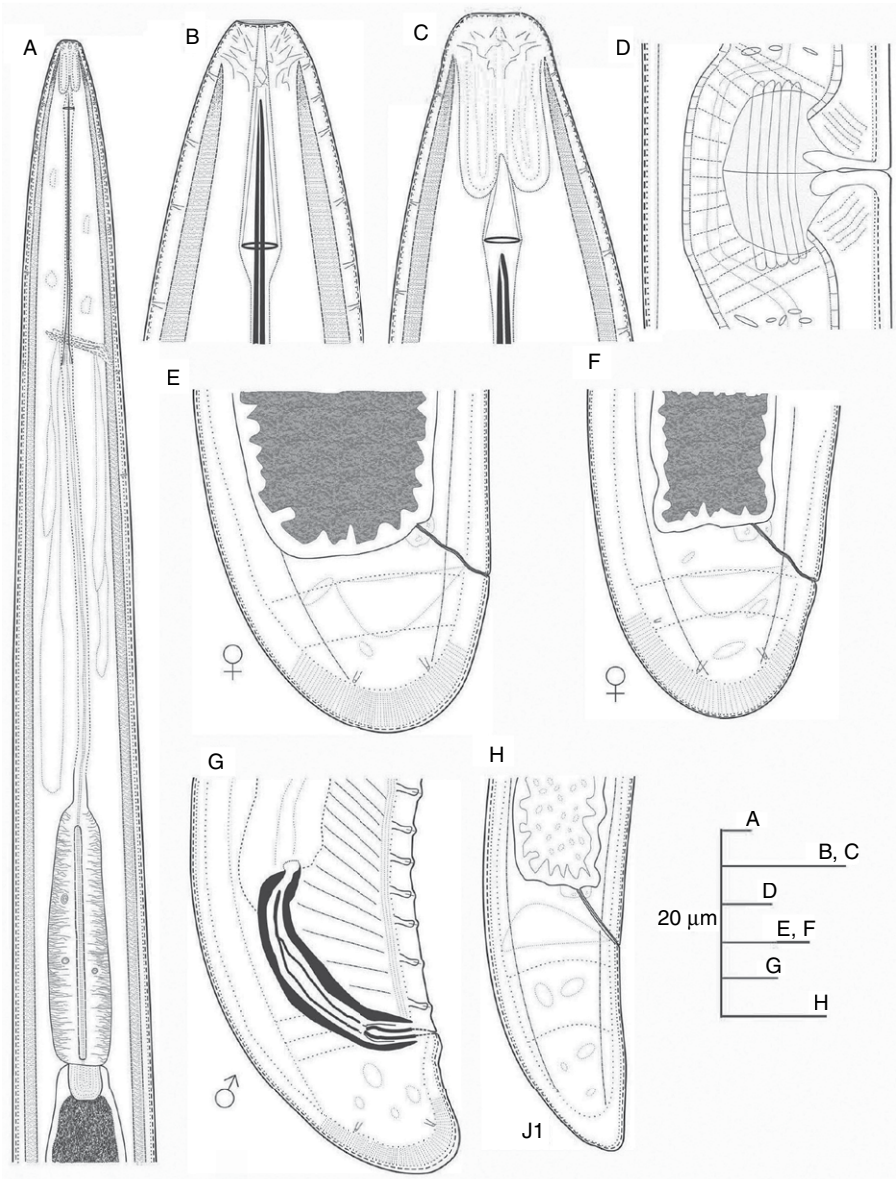

Fig. 7.9. An example of a nematode line drawing of *Longidorus onubensis* using a computer-aided program. (Reproduced from Archidona-Yuste *et al*., 2016.)

sit on its own layer, a transparent overlay effectively floating over the canvas. The fact that each element has its own layer means that editing is greatly simplified – merely select the appropriate layer and move, change or delete its content as appropriate. In a complex figure, there may be well in excess of 20 layers 'stacked' above the canvas. The file can be saved with all layers intact (in which case the file extension may belong to the program, e.g. PSD for Photoshop), thereby facilitating later changes that may be necessary after the paper has been submitted and reviewed; for example, lettering can be removed or altered without affecting an image on the other layers and without the need for erasing and/or background cloning. Files with the layers preserved are substantially larger than those where they have been compressed or flattened, so it is best if

authors retain a file with all the layers intact for archive purposes, but to flatten the layers and save appropriately as TIFF or JPEG files when submitting to a journal.

7.4.2.8 Resolution and apparent image quality

Inappropriate resolution is one of the most frequently encountered problems with images submitted for publication. Typically, an image for reproduction in a book or journal should be at a minimum of 300 dpi for greyscale or colour and 600–800 dpi for line drawings (some journals stipulate 1000 dpi). A common problem is caused by authors submitting files with a small canvas size and a resolution of 96 dpi or less. Part of the reason for this error is perceived image sharpness or quality. A typical computer screen has a resolution of 100 dpi or less – any image with a resolution higher than this does not improve the screen appearance from a typical viewing distance and so a 96 dpi image will probably look fine at normal viewing distances and magnification. Printed images, on the other hand require much higher resolution to avoid obvious pixelation, in part because a printed page is normally viewed at much closer distances than a monitor (the apparent sharpness, or acutance, of an image is subjective and influenced by several factors, including viewing distance, contrast and edge effects). Many such problems can be avoided before submission to a journal by first printing out the image and checking for appearance, rather than relying on the monitor image as a guide to quality. Judicious post-processing using an unsharp mask can increase apparent sharpness by adjusting (masking) the edge effect, but too much sharpening will actually achieve the opposite result to that intended. It is always advisable to check the requirements of the journal as these may vary considerably in terms of resolution, file type, etc., and files that fail to meet the required standards may be rejected.

A second problem that may arise during image processing involves the relationship between canvas size (physical dimension) and number of pixels (resolution). The characteristics of a digital image depend on the type of device used (such as a scanner or camera) and the way in which image quality can be customized, perhaps by specifying physical dimension and resolution beforehand rather than relying upon auto decisions by the device software. For example, a scanner, left to its own devices, may produce an image 100 cm across at a resolution of 72 dpi. Using an image processing program, a user reduces the image size to the desired size, say, 15 cm across and saves the file. The problem now is that the resolution of the physically much smaller image is still at 72 dpi – nowhere near high enough except for monitor viewing or a PowerPoint presentation. Data have been lost and cannot be put back. An image 100 cm across at 72 dpi contains about 2800 pixels of information. If the image size is reduced to 15 cm, then the dpi can be simultaneously increased to about 480 dpi – no information has been lost and no 'empty' pixels have been added as the file size will be virtually identical, i.e. all the data/pixels in the original image have been effectively retained, just 'compressed' into a smaller space. In this example, increasing the base resolution to, for example, 600 dpi will result in 'empty' interpolation – more dpi, but no extra information. Changing the physical dimensions of an image and dpi must be done at the same time if maximum information is to be retained.

Images should be supplied as separate files saved in an appropriate format, not embedded in Word documents or PowerPoint as resolution down-sampling, not to mention distortion caused by adjusting image size with the aspect ratio unlocked and drifting labels that have not been anchored, can be a problem with these programs. PDF files, particularly for dendrograms and the like, are usually acceptable as long as the resolution has not been down sampled by the program. Printing images at either the page size for the journal or the intended final size should confirm whether quality is sufficient or not. This should be done before submission of the manuscript in order to avoid potential delays in sending the paper out for review.

7.5 Acknowledgement

We extend our grateful acknowledgement to David Hooper, parts of this section being modified and expanded from his own excellent chapter, *Drawing and measuring nematodes* (Hooper, 1986).

7.6 References

Archidona-Yuste, A., Navas-Cortés, J.A., Cantalapiedra-Navarrete, C., Palomares-Rius, J.E. and Castillo, P. (2016) Unravelling the biodiversity and molecular phylogeny of needle nematodes of the genus *Longidorus* (Nematoda: Longidoridae) in olive and a description of six new species. *PLoS One* 11, e0147689. DOI: 10.1371/journal.pone.0147689

Brown, D.J.F. (1981) The effect of operator and measuring system error on the morphometrical variability of one nematode specimen. *Revue de Nématologie* 41, 169–171.

Brown, D.J.F., Grunder, J., Hooper, D.J., Klingler, J. and Kunz, P. (1994) *Longidorus arthensis* sp. n. (Nematoda: Longidoridae) a vector of cherry rosette disease caused by a new nepovirus in cherry trees in Switzerland. *Nematologica* 40, 133–149. DOI: 10.1163/003525994X00094

Clark, W.C. (1963) A new species of *Trichodorus* (Nematoda: Enoplida) from Westland, New Zealand. *New Zealand Journal of Science* 6, 414–417.

Cobb, N.A. (1890) A nematode formula. *Agricultural Gazette of New South Wales* 1, 131–136.

De Grisse, A. (1964) Morphological observations on *Criconemoides*, with a description of four new species found in Belgium (Nematoda). *Mededelingen Landbouhogesch Opzoek Staat Gent* 29, 734–761.

de Man, J.G. (1880) Die Einheimischen, frei in der reinen Erde und im stissen Wasser lebenden Nematoden. *Vorlaufiger Bericht und descriptiv-systematischer Theil. Tijdschrift Nederlandsch Dierkunde Vereen* 5, 1–104.

Decraemer, W., Cantalapiedra-Navarrete, C., Archidona-Yuste, A., Varela-Benavides, I., Gutiérrez-Gutiérrez, C., Castillo, P. and Palomares-Rius, J.E. (2019) Integrative taxonomy unravels cryptic diversity in the *Paratrichodorus hispanus*-group complex and resolves two new species of the genus and the molecular phylogeny of the family (Nematoda: Trichodoridae). *Zoological Journal of the Linnean Society* 185, 656–692. DOI: 10.1093/zoolinnean/zly059

Frederick, J.J. and Tarjan, A.C. (1978) Variability in measurements made of same nematode specimen by various observers or by one observer on different days. *Nematologica* 24, 476–478. DOI: 10.1163/187529278X00641

Hooper, D.J. (1986) Drawing and measuring nematodes. In: Southey, J.F. (ed.). *Laboratory Methods for Work with Plant and Soil Nematodes*, 6th ed. Her Majesty's Stationery Office, London, UK, pp. 87–94.

Lišková, M., Robbins, R.T. and Brown, D.J.F. (1997) Descriptions of three new *Longidorus* species from Slovakia (Nemata: Longidoridae). *Journal of Nematology* 29, 336–348.

Loof, P.A.A. (1969) Taxonomy of some species of the genus *Mesodorylaimus* Andrássy, 1959 (Dorylaimoidea). *Nematologica* 15, 253–274. DOI: 10.1163/187529269X00687

Loof, P.A.A. and Coomans, A. (1970) On the development and location of the oesophageal gland nuclei in the Dorylaimina. *Proceedings of the IX International Nematology Symposium (Warsaw, 1967)*, pp. 79–161.

Loof, P.A.A. and Coomans, A. (1972) The oesophageal gland nuclei of Longidoridae (Dorylaimida). *Nematologica* 18, 213–233. DOI: 10.1163/187529272X00458

Mehta, U.K. and Raski, D.J. (1971) Revision of the genus *Criconema* Hoffmanner and Menzel, 1914 and other related genera (Criconematidae: Nematoda). *Indian Journal of Nematology* 1, 145–198.

Nguyen, K.B. and Hunt, D.J. (eds). (2007) *Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts*. In: *Nematology Monographs and Perspectives, 5* (Series Editors: Hunt, D.J. & Perry, R.N.). Brill, Leiden, The Netherlands.

Subbotin, S.A., Mundo-Ocampo, M. and Baldwin, J.G. (2010) *Systematics of Cyst Nematodes (Nematoda: Heteroderinae)*. In: *Nematology Monographs and Perspectives,* 8A. (Series Editors: Hunt, D.J. and Perry, R.N.). Brill, Leiden, The Netherlands.

Ye, W. (1996) Applying Microsoft Works spreadsheet in statistics for morphometric data of nematode identification. *Afro-Asian Journal of Nematology* 6, 203–211.

Zancada, M.C., Mahajan, R. and Sánchez, A. (1987) Evaluation of characters used to identify species of the genus *Rotylenchus* Filipjev, 1936 (Nematoda: Hoplolaimidae). *Nematologica* 33, 286–297. DOI: 10.1163/187529287X0041

8 Electron Microscopy Techniques

VLADIMIR V. YUSHIN,[1] MYRIAM CLAEYS,[2] JAN L.M. LEUNISSEN[2] AND JULIA K. ZOGRAF[1]

[1]*National Scientific Center of Marine Biology, Vladivostok, Russia;* [2]*Department of Biology, Ghent University, Belgium*

8.1 Transmission Electron Microscopy

Transmission electron microscopy (TEM) is one of the routine methods in studies of tissues and cells of all organisms and has been used extensively in nematology for various studies, including ultrastructural information of nematodes, morphogenesis, nematode-host interactions, responses to biotic and abiotic effects, and as an important adjunct to taxonomic information (e.g. Bird, 1971, 1984; McLaren, 1976; White *et al*., 1986; Bird and Bird, 1991; Wright, 1991; Malakhov, 1994; Endo, 1998; Justine, 2002; Yushin *et al*., 2002, 2016; Hall and Altun, 2008; Müller-Reichert *et al*., 2008; Giblin-Davis *et al*., 2010; Hall *et al*., 2012; Basyoni and Rizk, 2016; Mulcahy *et al*., 2018). The literature on TEM methods is extensive and includes several handbooks and reviews (Glauert and Lewis, 1998; Hajibagheri, 1999; Bozzolla and Russell, 1999; Hayat, 2000; Kuo, 2014) and internet sites (e.g. http://www.wormatlas.org/methods.htm). The methods of TEM as applied to nematodes have been elaborated in detail and are available with comprehensive descriptions of each processing step (Bird, 1971; Shepherd and Clark, 1986; Carta, 1991; Hall, 1995; Hall *et al*., 2012; Serwas and Dammermann, 2015; Mulcahy *et al*., 2018). The basic information from these sources is strongly recommended for all nematologists who plan to study the ultrastructure of soil and plant-parasitic nematodes.

Nematode specimens fixed for TEM and embedded into resins are a goldmine for observations by a variety of modern methods (Bumbarger *et al*., 2009; Müller-Reichert *et al*., 2010; Hall *et al*., 2012; Kolotuev *et al*., 2012; Hall and Rice, 2015; Manning and Richmond, 2015; Serwas and Dammermann, 2015; Bert *et al*., 2016; Yushin *et al*., 2016; Zhang *et al*., 2016; König *et al*., 2017; Li *et al*., 2017; Han *et al*., 2018; Mulcahy *et al*., 2018). The success of analysis of EM results depends strongly on the method of preparation, which includes uniform successive steps of tissue processing common for all organisms, i.e. primary fixation by aldehydes and post-fixation by osmium tetroxide, dehydration, infiltration and embedding into resin. In this chapter we have not included descriptions of general principles of fixation and embedding or theoretical details of further preparation for conventional TEM; this information is readily available in comprehensive books and guides detailing extensive experience in studies of animal tissues (e.g. Weakley, 1981; Bozzolla and Russell, 1999; Hayat, 2000; Kuo, 2014). Here we provide a newcomer with the definitive TEM protocols tested on a variety of soil, plant and fresh-water nematodes, which are suitable for initial studies. Modern methods of TEM techniques and observations that have been developed after the review of Shepherd and Clark (1986) will be outlined briefly. The chemical processing of nematodes for TEM has been improved to take into account the low permeability of nematode cuticle and tissues, resulting in numerous protocols, each designed for specific requirements, such as nematode species, stage, tissue, etc. For novices we propose a

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
10.1079/9781786391759.0008

straightforward way of chemical preparation of nematodes from fixation to epoxy plastic blocks ready for ultramicrotomy (Table 8.1). This protocol has been tested on soil, plant and fresh-water nematodes by the authors of this section and may be proposed as the basis for new improved versions.

8.1.1 Before fixation

In the case of soil and plant-parasitic nematodes the main difficulties are the small size of the adults and juveniles and impermeability of the body cuticle to chemicals used for TEM fixation and embedding. Glass

Table 8.1. Processing specimens for conventional TEM.

PRIMARY FIXATION	
Fixative A Glutaraldehyde-paraformaldehyde (GA-PF) (10 ml)	**Fixative B** Glutaraldehyde (GA) (10 ml)
1 ml distilled water (DW) 2.5 ml cacodylate buffer (CB) (0.2 M, pH = 7.2–7.4) 0.5 ml 0.5% MCl_2 1 ml 25% GA (EM Grade) 5 ml 4% PF	6 ml DW 2.5 ml CB 0.5 ml 0.5% MCl_2 1 ml 25% GA
Fixative content: 2.5% GA, 2% PF, 0.05 M CB, 0.025% MCl_2	Fixative content: 2.5% GA, 0.05 M CB, 0.025% MCl_2
1. **Relaxation** by cold at 4°C 2. **Prefixation** before cut (from several minutes to many hours depending on the nematode species). 3. **Cut and fix** in a fresh portion of the fixative at 4°C overnight.	
4. **Rinsing** (rinsing buffer (20 ml): 5 ml CB + 15 ml DW) at least three times at 4°C (15 min + 1 h + 3–4 h or overnight). Series of fixed nematodes may be collected and stored up to 2 weeks in rinsing buffer at 4°C without significant deterioration of final results.	
SECONDARY FIXATION	
Fixative 2 Osmium tetroxide (OsO_4) 4 ml: 1 ml CB + 2 ml DW + 1 ml 4% OsO_4 = Fixative content: 1% OsO_4, 0.05 M CB	
5. **Osmium tetroxide fixation** 1–4 h, room temperature. Shaking or rotation is recommended. 6. **Rinsing:** rinsing buffer (once), distilled water (at least three times: 15 min + 1 h + 3 h). 7. ***En bloc* staining** in 1% aqueous solution of uranyl acetate (UA) for 1 h (full darkness, room temperature, rotation). 8. **Rinsing** in DW, twice.	
DEHYDRATION	
9. Ethanol and isopropanol series, 5 min each: Ethanol, percentage: 7-15-30-50-70-90-100 – (mixture 1 ethanol : 1 isopropanol) - pure isopropanol (twice)	
INFILTRATION	
Spurr's low viscosity resin	**Epon-like resin**
10. **Infiltration** in graded mixtures of Spurr's resin (standard hardness) with isopropanol, room temperature, rotation: 1 Spurr: 3 isopropanol – 1 h; 1:1 – 2 h; 3:1 – 4 h. 11. **Infiltration in pure Spurr's** at 4°C, overnight.	10. **Infiltration** by Epon without accelerator (DMP-30) diluted in isopropanol, room temperature, rotation: 1 Epon: 3 isopropanol – 1 h 1:1 – 2 h; 3 Epon *with accelerator* (DMP-30)/1 isopropanol, overnight at 4°C. 11. **Infiltration in pure Epon** at 4°C, overnight.
12. **Embedding** in fresh portion of epoxy resin in embedding moulds, polymerization at 60°C, 1–2 days.	

embryo dishes are convenient as a bath for preliminary operations with nematodes, i.e. collection, experimental treatment, heat relaxation, prefixation and cutting. All subsequent steps of chemical processing may be easily carried out in 1.5 ml Eppendorf tube or freezing vials with screw caps. In this case successive chemicals can be replaced using glass Pasteur pipettes under a dissecting microscope.

8.1.2 Primary fixation

Two versions of aldehyde fixers are used for preparation of TEM both with good, predictable results. A mixture of glutaraldehyde (2.5%) with paraformaldehyde (1–4%) is traditionally used in TEM. Glutaraldehyde on its own often gives results that are better than the aldehyde mixtures and it is recommended to test both methods.

Sodium cacodylate buffer is recommended for fixative composition as preparation is straightforward and stock solutions remain stable for a long time. Phosphate buffer is physiologically more appropriate and has been used successfully (Carta, 1991). Magnesium chloride ($MgCl_2$) or calcium chloride ($CaCl_2$) are normally used as an additive for best preservation of membranes (Hayat, 2000).

The aldehyde fixatives proposed in Table 8.1 are hyperosmotic to tissues of plant, soil and fresh-water nematodes. This is not the case for animal-parasitic nematodes, especially marine species, whose tissues have a higher osmotic pressure and thus the fixatives must be adjusted by sodium chloride (NaCl) or sucrose (Hayat, 2000; Yushin *et al*., 2002).

8.1.3 Chemical fixation

The technique of initial processing of nematodes includes relaxation, prefixation and cutting. There are different ways of relaxation but the most physiologically neutral relaxation by cold (incubation at 4°C) is preferable (Shepherd and Clark, 1986; Carta, 1991; Hall, 1995). Prefixation by cold aldehyde fixer immobilizes nematodes and makes them suitable for cutting by avoiding explosive release of the internal turgor pressure and resulting tissue damage. The period of prefixation depends on the nematode species and varies from several minutes to many hours (Bird, 1971; Shepherd and Clark, 1986; Carta, 1991).

Piercing or cutting are necessary before following TEM processing of prefixed nematodes. Various techniques have been described (Bird, 1971; Carta, 1991; Hall, 1995). We found an easy method to achieve accurate cutting by using the sharp edge of a glass capillary tube, which was first cut in the middle using a diamond needle file and then broken in two; using this transparent 'glass knife' makes 'microsurgery' in an embryo dish under a dissecting microscope fast and easy. After cutting the nematodes, specimens must be transferred into a vial filled with fresh aldehyde fixative. From this point, all treatment of specimens must be carried out in capped vials agitating in a rotary mixer or vortex shaker. For the following processes, the specimens may be embedded into agar or agarose blocks, thus making replacements of solutions easier (Shepherd and Clark, 1986; Hall, 1995).

The period needed to achieve high-quality aldehyde fixation depends on the species, but is usually from 1 h to overnight, although it may be longer. Long-term storage in fixative is not recommended. Rinsing in pure buffer after aldehyde fixation is a very important procedure to avoid precipitation of traces of aldehydes in tissues. Specimens must be washed in several changes of rinsing buffer with incubation, for example, overnight (Shepherd and Clark, 1986; Hall, 1995). Washed specimens may be stored for several weeks in rinsing buffer at 4°C before being used for further processing, without major ultrastructural alterations; however, if possible long-term storage is best avoided.

Before secondary fixation by osmium tetroxide, the specimens must be transferred into a new, clean vial to ensure there are no traces of aldehyde. The time of the secondary fixation may be relatively short (but at least 1 h), and using a rotary shaker is strongly recommended. Rinsing specimens after osmium tetroxide fixation includes incubation in several changes of distilled water.

Tertiary fixation (staining) of specimens *en bloc* in 0.5–1.0% aqueous solution of uranyl acetate solution improves ultrastructural definition and makes staining of sections much easier (Carta, 1991; Glauert and Lewis, 1998). This procedure is strongly recommended before embedding into the low viscosity Spurr's resin (Spurr, 1969), which is nearly impermeable to uranyl acetate.

8.1.4 Infiltration and embedding: epoxy resins

Two main types of epoxy resins, Epon-like media (several new brands related to basic Epon or Araldite) and Spurr's resin, are usually used for embedding specimens for TEM (Carta, 1991; Glauert and Lewis, 1998; Hall *et al*., 2012). Spurr's resin is excellent for infiltration of nematodes and plant tissues due to its low viscosity, making manipulations and embedding easy. However, Spurr's resin is hygroscopic and quality deteriorates when it absorbs atmospheric water, so exposure of vial contents to air must be as brief as possible. The viscous Epon-like (or Araldite) resins are less easy, or even impossible, to use because of the need for long-term impregnation, with occasional embedding failures. However, tissues successfully infiltrated with Epon usually show better preservation and contrast of subcellular details and less extraction of cell contents when compared with specimens embedded in Spurr's resin.

Before infiltration into resin, the specimens must be dehydrated in organic solvents. Dehydration may be processed in through an ethanol series finishing with absolute ethanol, acetone or propylenoxide. From our experience the less volatile isopropanol (isopropyl alcohol) was more successful as a solvent than acetone or propylenoxide, due mainly to less extraction of cell contents during infiltration, especially when Spurr's resin is used.

Infiltration requires step-by-step substitution of solvent by embedding medium, with the final incubation of specimens in pure resin. The period of infiltration in Spurr's resin is much shorter than in Epon resin. If infiltration is done in vials, it is recommended that the process of intermixing of the viscous layers is done on a rotary shaker. The specimens are usually concentrated at the conical tip of the vial, so must be moved to the upper layer of the medium using a glass Pasteur pipette.

After infiltration, nematodes are embedded in pure resin using latex, plastic or silicone embedding moulds (Bozzolla and Russell, 1999). The hygroscopic silicone moulds are unsuitable for embedding into Spurr's resin.

Caution: The fixatives (glutaraldehyde, paraformaldehyde, osmium tetroxide), solvents (acetone, propylene oxide, isopropanol), embedding media and stains (uranyl acetate, lead citrate) used for preparation of specimen for conventional TEM may be dangerous, i.e. carcinogenic, mutagenic, toxic or harmful. Warnings for each chemical on factory packaging and precautions in guides must be carefully studied before starting work (Glauert and Lewis, 1998; Bozzolla and Russell, 1999). Working in a fume hood and wearing hand protection is undeniably necessary.

8.1.5 Specimen preparation: acrylic resins

The hydrophilic acrylic resins, such as JB-4, LR White, LR Gold and Lowicryl, may be recommended as embedding media for morphological and immunocytochemical studies when ultrastructural details are not of paramount importance (Glauert and Lewis, 1998; Newman and Hobot, 1999; Yeung and Huang, 2015). The reduced picture quality, when compared with epoxy resins, is offset by low toxicity, the omission of several steps of specimen preparation (osmium tetroxide fixation, uranyl acetate *en bloc* stain), plus rapid and excellent infiltration and embedding. Acrylic resins retain the protein antigenicity of tissues much better than epoxy resins, so are used widely for post-embedding immunochemistry (Hajibagheri, 1999; McDonald, 2014a). Procedures for acrylic resins have been described and discussed in several guides (Hall, 1995; Glauert and Lewis, 1998; Newman and Hobot, 1999; Hall *et al*., 2012; Yeung and Huang, 2015). The straightforward and well-tested schedule of specimen preparation using LR White resin as the embedding medium is outlined in Table 8.2. Specimens can be prepared easily for both conventional TEM observations and post-embedding immunocytochemistry.

Primary procedures of nematode fixation in general are the same as for epoxy resins (see Sections 8.1.1–8.3). For morphological observations each of the common aldehyde fixations (Table 8.1) may be chosen. The following fixation procedures may be omitted or limited to 20–30 min incubation in 0.2–0.5% aqueous uranyl acetate (recommended). Fast infiltration and embedding into LR White resin with subsequent polymerization at 60°C may condense total duration of specimen preparation from fixation to polymerized blocks to several hours (Bozzolla and Russell, 1999). The final result differs from the standard epoxy resin embedded specimens; however, LR White procedures as less hazardous and time consuming, and may be recommended for routine experiments (Fig. 8.1).

Table 8.2. Processing specimens for LR White embedding. (Abbreviations as for Table 8.1.)

1. **Aldehyde fixation.** Fixative for immunochemistry (PF-GA, mixture for 10 ml): 1.5 ml DW 2.5 ml CB (0.2 M, pH = 7.2–7.4) 0.5 ml 0.5% MCl_2 0.5 ml 8% GA (EM Grade) 5 ml 4% PF Fixative: 2% PF, 0.4% GA, 0.05M CB, 0.025% MCl_2. 1–3 h at 4°C.
2. **Rinsing in buffer overnight.** Before dehydration specimens must be rinsed in DW and incubated 1 h in 0.2% water solution of uranyl acetate.
3. **Dehydration.** Ethanol series, 3 min each: 5-10-15-30-50-70-80-90-96%.
4. **Infiltration at 4°C.** Mixtures of LR White with 96% ethanol, 4°C. 1 LRW: 3 ethanol – 1 h; 1:1 – 2 h; 3:1 – 4 h.
5. **Infiltration in pure LR** White at 4°C, overnight.
6. **Embedding** in closed gelatinous capsules. Polymerization for routine TEM: 50–60°C, 1 day; for post-embedding immunolabelling: 37°C, 1 week.

Fixatives based on paraformaldehyde in buffer with no, or very low, concentration of glutaraldehyde are preferable for immunocytochemistry (Hall, 1995; Glauert and Lewis, 1998; Newman and Hobot, 1999). The total duration of fixation must be relatively short (30 min) to retain protein immunoreactivity.

It is strongly recommended for nematodes fixed only with aldehydes to start dehydration in a low concentration of ethanol to prevent deformation. After gradual dehydration and infiltration, specimens must be embedded inside gelatinous capsules, each closed by a cap to isolate the embedding medium from atmospheric oxygen. Polymerization of blocks with specimens for further post-embedding immunolabelling must be done slowly at lower temperature (37°C), or cold (4°C) using UV irradiation (Hall, 1995; Hall *et al.*, 2012). The specimens fixed as outlined in Table 8.2 are suitable both for morphological observations and immunogold labelling (Fig 8.2).

8.1.6 Cryopreparation methods for TEM

Conventional chemical fixation and embedding, as the standard methods for morphological studies of nematode tissues by TEM, are suitable for most studies of soil and plant-parasitic nematodes, despite several limitations including artefacts caused by hyperosmotic fixatives and loss of immunoreactivity (Rostaing *et al.*, 2004). Successful fixation of nematodes with their highly protective cuticle is impossible without laborious cutting of each individual specimen with inevitable structural degradation (Bert *et al.*, 2016). The useful method of fixation without surgery is microwave-assisted fixation, which is well established and described (Jones and ap Gwynn, 1991; Hall *et al.*, 2012). In comparison with the classical approach, microwave-assisted fixation enables not only improved general ultrastructural observations, but also provides for improved post-embedding immunocytochemistry (Wergin *et al.*, 2000; Hall *et al.*, 2012; Webster, 2014).

The rapid combined physical and chemical fixation of specimens achieved using cryomethods is, however, indispensable for high-resolution studies involving immunocytochemistry, electron tomography and three-dimensional (3D) reconstructions, quantitative assessments of cytoplasmic elements, and correlative light and electron microscopy (CLEM) (Hurbain and Sachse, 2011; McDonald, 2014b; Mielanczyk *et al.*, 2014; Bert *et al.*, 2016; Mulcahy *et al.*, 2018). High-pressure freezing with subsequent freeze substitution (HPF/FS) is widely used in cell and developmental biology especially with *C. elegans* (Claeys *et al.*, 2004; Rostaing *et al.*, 2004; Weimer, 2006; McDonald, 2007, 2014b; Hall *et al.*, 2012; Manning and Richmond, 2015; Serwas and Dammermann, 2015; Mulcahy *et al.*, 2018). Cryofixation and cryopreparation of

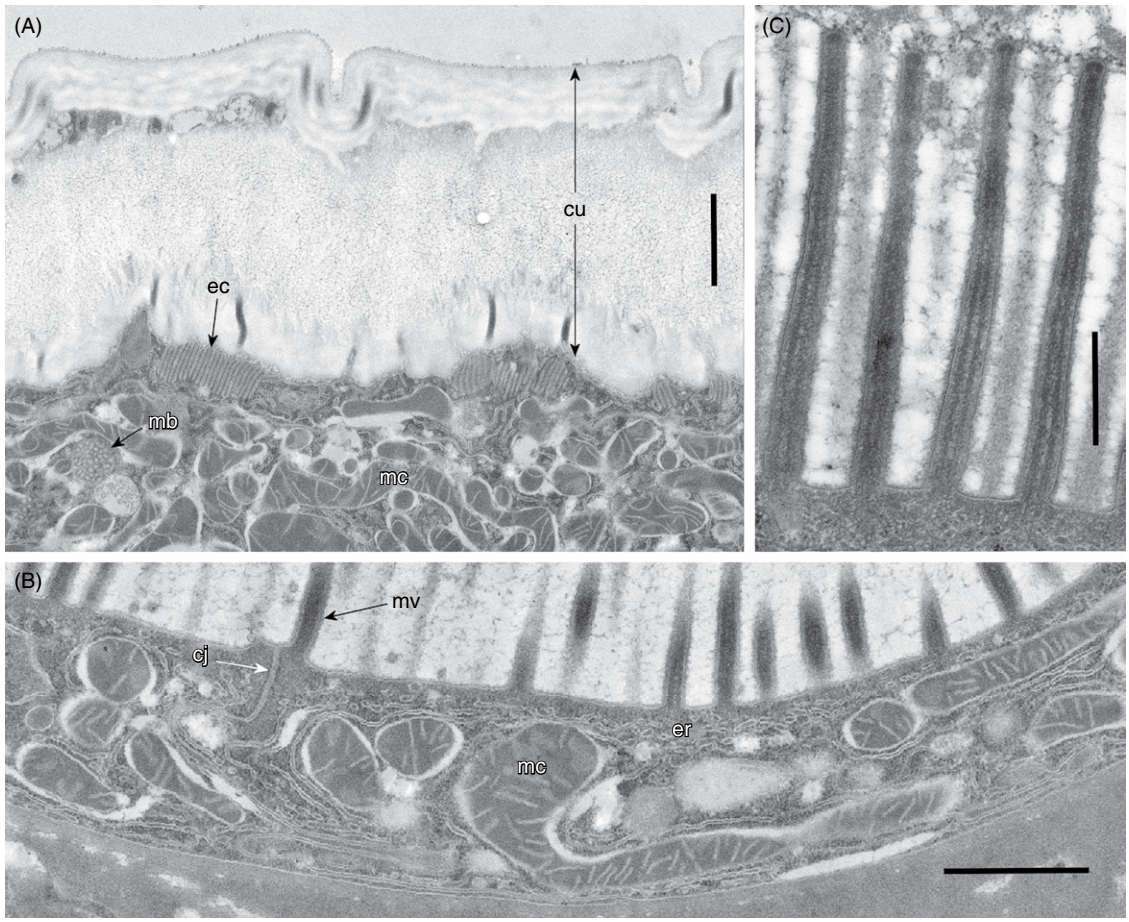

Fig. 8.1. *Leidynema portentosae* (Rhabditida, Thelastomatidae), scanning transmission electron microscopy (STEM) by Zeiss Sigma 300 VP. Morphological paraformaldehyde-glutaraldehyde fixation without osmium tetroxide and uranyl acetate post-fixations; embedding in acrylic LR White resin (see Table 8.2, polymerization at 60°C), double-stained sections (uranyl acetate and lead citrate, Table 8.6). **(A)** Body cuticle and ectoderm filled with mitochondria. **(B)** Intestinal epithelium. **(C)** Microvilli of intestinal epithelium at higher magnification. Abbreviations: cj, cell junction; cu, cuticle; ec, microvilli-like complication of epidermal surface; er, endoplasmic reticulum; mb, multivesicular body; mc, mitochondria; mv, intestinal microvilli. Scale bars: A, B = 1 µm; C = 0.5 µm.

individuals *in toto* preserve structures and the arrangement of cellular components with fewer artefacts in cell and organelle morphological studies (Figs 8.3A and 8.4). This is especially important if the specimens are being prepared for 3D reconstructions, electron tomography or CLEM (Bumbarger *et al*., 2006; Müller-Reichert *et al*., 2007, 2010; Kolotuev *et al*., 2012; Hall and Rice, 2015; Serwas and Dammermann, 2015; Mulcahy *et al*., 2018). The cryopreparation methods are also the best way to preserve tissue epitopes for immuno-electron microscopy (IEM) (Rostaing *et al*., 2004; Weimer, 2006; Morphew, 2007; Hall *et al*., 2012; Yushin *et al*., 2016). For plant nematology, cryomethods are powerful when the study includes preparation of host plant tissues, which are impermeable to TEM chemicals (Karahara and Kang, 2014; Baranowski *et al*., 2018). Nematodes in general are well suited for fixation by HPF due to their dimensions, and have advantages for studies not only of whole animals (Fig. 8.3A), but also of embryos inside an eggshell with limited permeability (Hall *et al*., 2012; Serwas and Dammermann, 2015; Yushin *et al*., 2016).

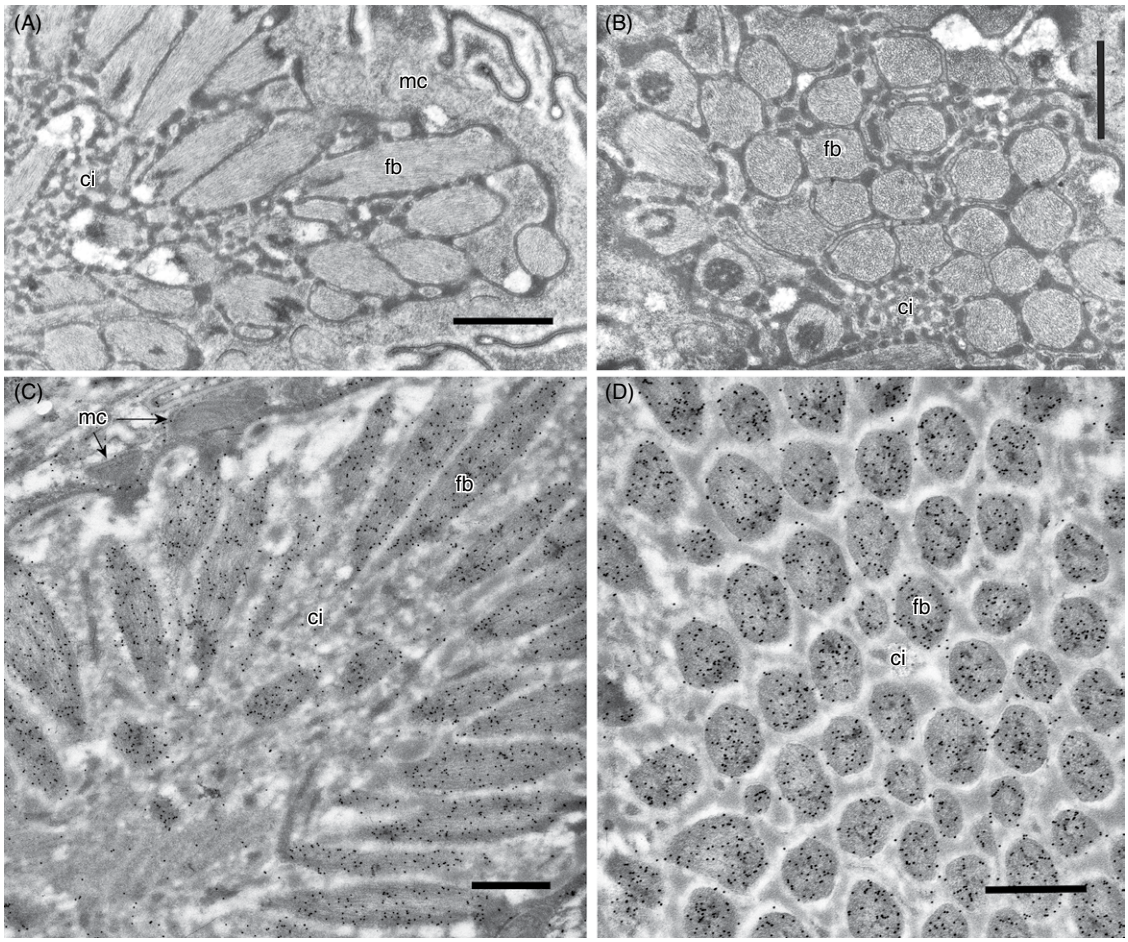

Fig. 8.2. *Sabatieria palmaris* (Araeolaimida, Comesomatida), immature spermatozoa from testis, TEM. Sections through the spermatozoa show specific fibrous bodies cut longitudinally **(A, C)** and transversely **(B, D)**. **(A, B)** Epoxy resin (Araldite) embedded specimen fixed by glutaraldehyde, post-fixed with osmium tetroxide and uranyl acetate (Table 8.1); double stain of sections with uranyl acetate and lead citrate. **(C, D)** Acrylic resin (LR White) embedded specimens fixed by paraformaldehyde-glutaraldehyde mixture (Table 8.2, polymerization at 37°C); sections are labelled with the antibody to major sperm protein (MSP) conjugated with colloidal gold (immunogold labelling, for details see Yushin *et al.*, 2016); double stain with uranyl acetate and lead citrate. Note intense labelling in fibrous bodies; labelling is excluded from membranous cisternae surrounding the fibrous bodies. Abbreviations: ci, membranous cisternae; fb, fibrous bodies; mc, mitochondria. Scale bars = 0.5 µm.

However, the decision to use HPF/FS and other methods based on cryofixation had to be justified for each project until recently. Time consumption and cost factors had serious impact on these decisions. The expensive HPF machines from Leica (EMPact and EMPact 2, HPM100 now replaced by Leica ICE), RMC Boeckeler (or Balzers) HPM 010 and Wohlwend Compact 03 are not widely distributed in nematology laboratories and institutions (Weimer, 2006; McDonald, 2014b). Specimens that have been cryofixed for study by conventional microtomy and microscopy need to be dehydrated by freeze substitution for hours up to several days before impregnation and embedding (Weimer, 2006; McDonald, 2014b), also processing and staining need to be adapted for each species or type of experiment. Some of these problems are reduced by development of an alternative cryofixation method (Leunissen and Yi, 2009; Han *et al.*, 2012; Grabenbauer *et al.*, 2014; Claeys *et al.*, 2017, 2019; Huebinger and Grabenbauer, 2018; see Section 8.1.9).

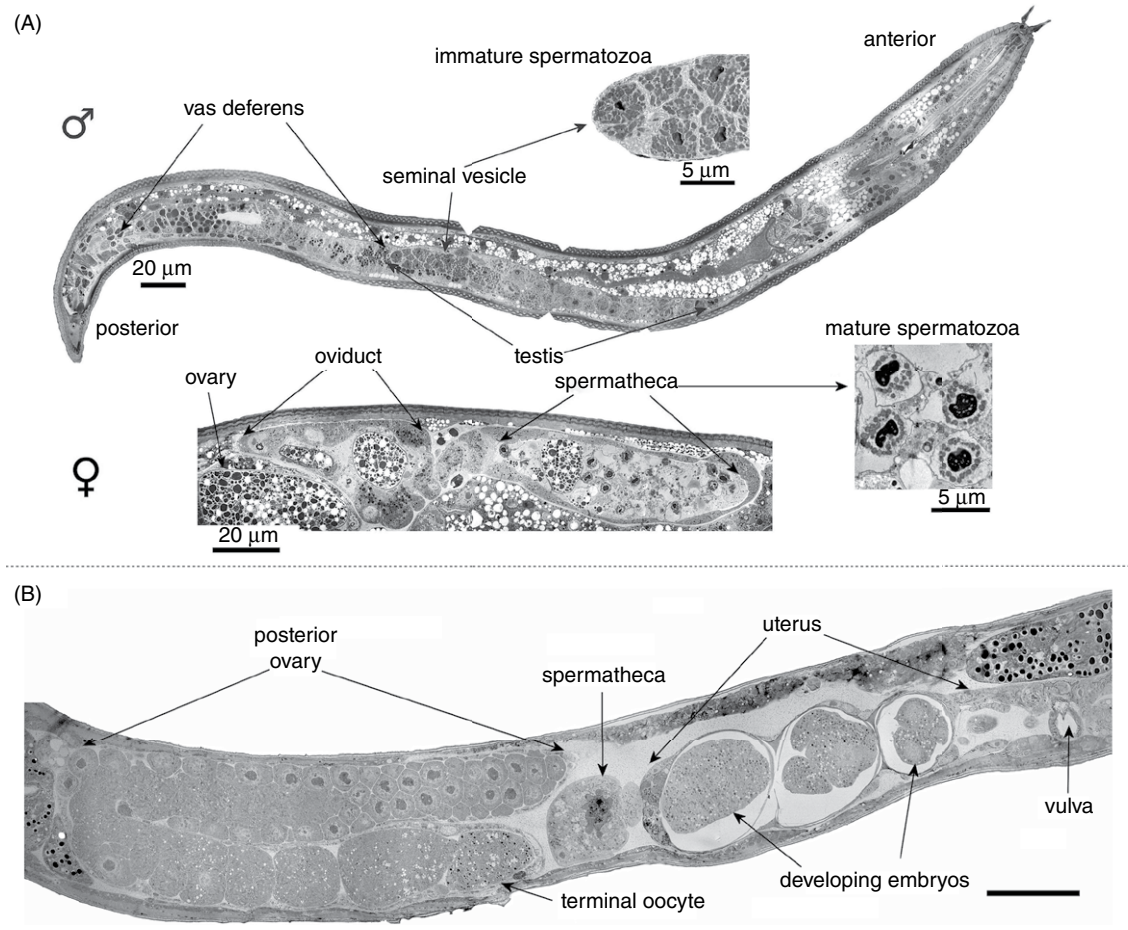

Fig. 8.3. Whole nematodes cryofixed and embedded without cutting, piercing or chemical enhancement of cuticle permeability. **(A)** *Acrobeles complexus*. TEM after HPF/AFS fixation (Tables 8.3 and 8.5) and LR White embedding, longitudinal sections through male (♂) and female (♀), double stain with uranyl acetate and lead citrate. Male: longitudinal section through the whole animal showing the position and structure of the male reproductive system and testis with immature spermatozoa. Female: part of the longitudinal section showing ovary, oviduct and spermatheca filled with mature spermatozoa. **(B)** *Caenorhabditis elegans.* TEM after SPRF/AFS fixation (Tables 8.4 and 8.5) and Spurr embedding. Part of the longitudinal section through the adult hermaphrodite, double stain with uranyl acetate and lead citrate. General view of the full posterior branch of the reproductive system showing ovary, spermatheca and uterus. Scale bar: 20 µm. (A, from Yushin *et al*., 2016; B, from Claeys *et al*., 2017.)

8.1.7 High-pressure freezing (HPF)

The general principles of cryopreparation for electron microscopy are available from several guides (e.g. Cavalier *et al*., 2008; McDonald, 1999, 2007; McDonald *et al*., 2007) and the use of HPF/FS with *C. elegans* has been described and discussed in a series of excellent reviews (Weimer, 2006; McDonald, 2007; Hall *et al*., 2012; Manning and Richmond, 2015; Serwas and Dammermann, 2015), which may be recommended for plant and soil nematologists. Here we outline briefly our modifications of the HPF/FS, which have been used successfully with different fixations (for morphology and post-embedding immunocytochemistry) and embedding media (Spurr's resin and LR White) on plant-parasitic and soil nematodes using Leica EM Pact2 HPF and Leica automatic freeze substitution (AFS) equipment (Lak *et al*., 2015; Yushin *et al*., 2016; Claeys *et al*., 2017; Figs 8.3A, 8.4, 8.5A,B).

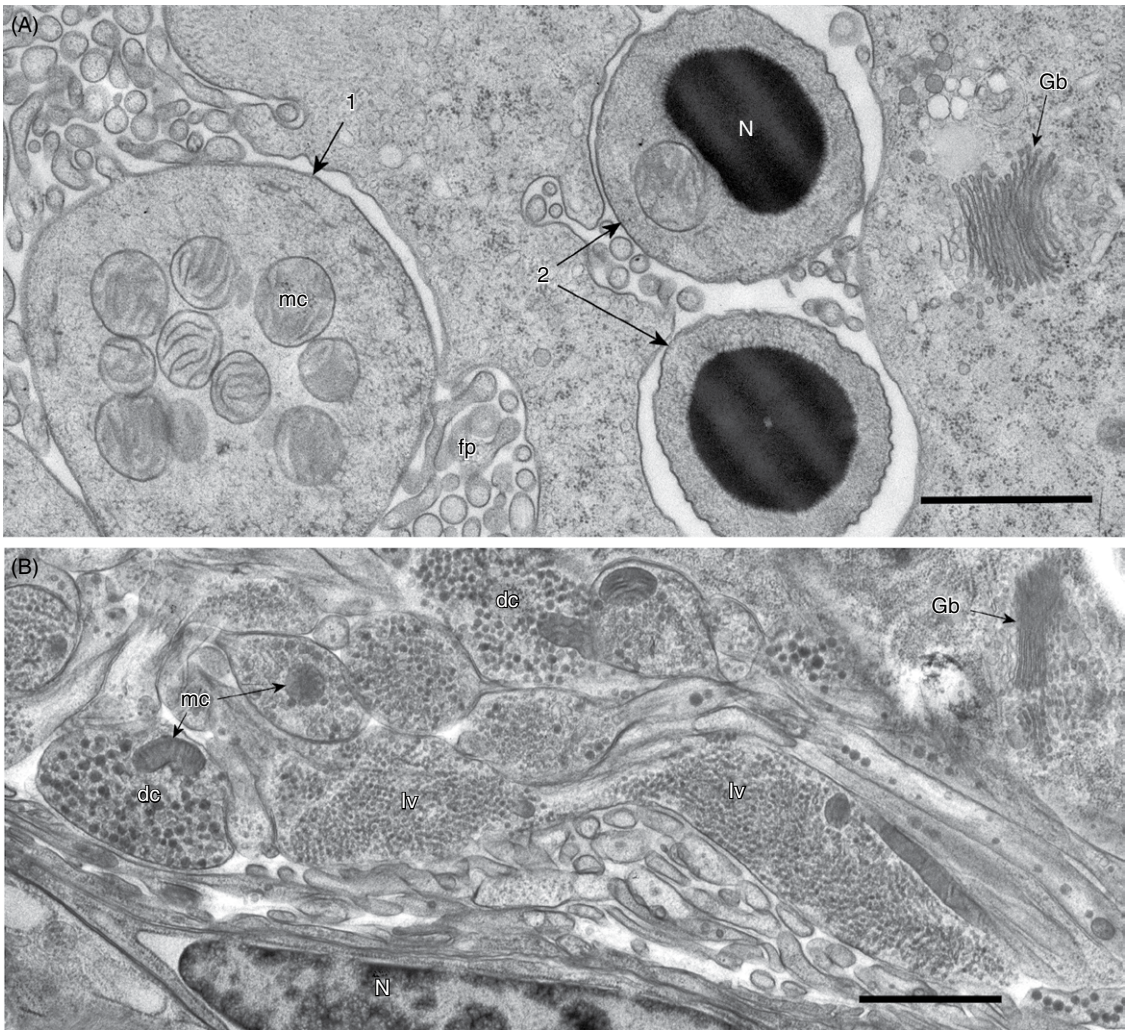

Fig. 8.4. *Mylonchulus* sp. (Mononchida, Mylonchulidae), TEM. HPF/AFS processing (AFS as in Table 8.5), Spurr embedding; double stain with uranyl acetate and lead citrate. **(A)** Spermatids in testis cross sectioned at the level of mitochondria (1) and nucleus (2). **(B)** Nerve ring; two types of synaptic vesicles (light and dark-core) fill the neuronal elements. Abbreviations: dc, dark-core synaptic vesicles; fp, filopodia; Gb, Golgi body; lv, light synaptic vesicles; mc, mitochondria; N, nucleus. Scale bars: 1 µm.

The aforementioned commercial HPF device basically consists of a pressure and a cooling system that are fully automatic. Both are connected by a shutter mechanism to coordinate pressurizing prior to freezing. By pressing the start button, the high-pressure freezing takes place. Once the sample had been ejected into a bath of liquid nitrogen (LN) the freeze substitution (FS) procedure can start.

Table 8.3 gives information on the procedures for HPF. The main goal of rapid freezing of nematodes is to preserve their ultrastructure in its physiologically natural state. This can be achieved only by accurate and well-planned work. Keep all the tools for loading close at hand and practice loading material into the freeze specimen holder before attempting to freeze real samples. The development of HPF devices results in significant changes between the previous and current models (Kaech and Ziegler, 2014). Follow the instruction manual carefully or contact the company that distributes the HPF device. Each equipment needs special training.

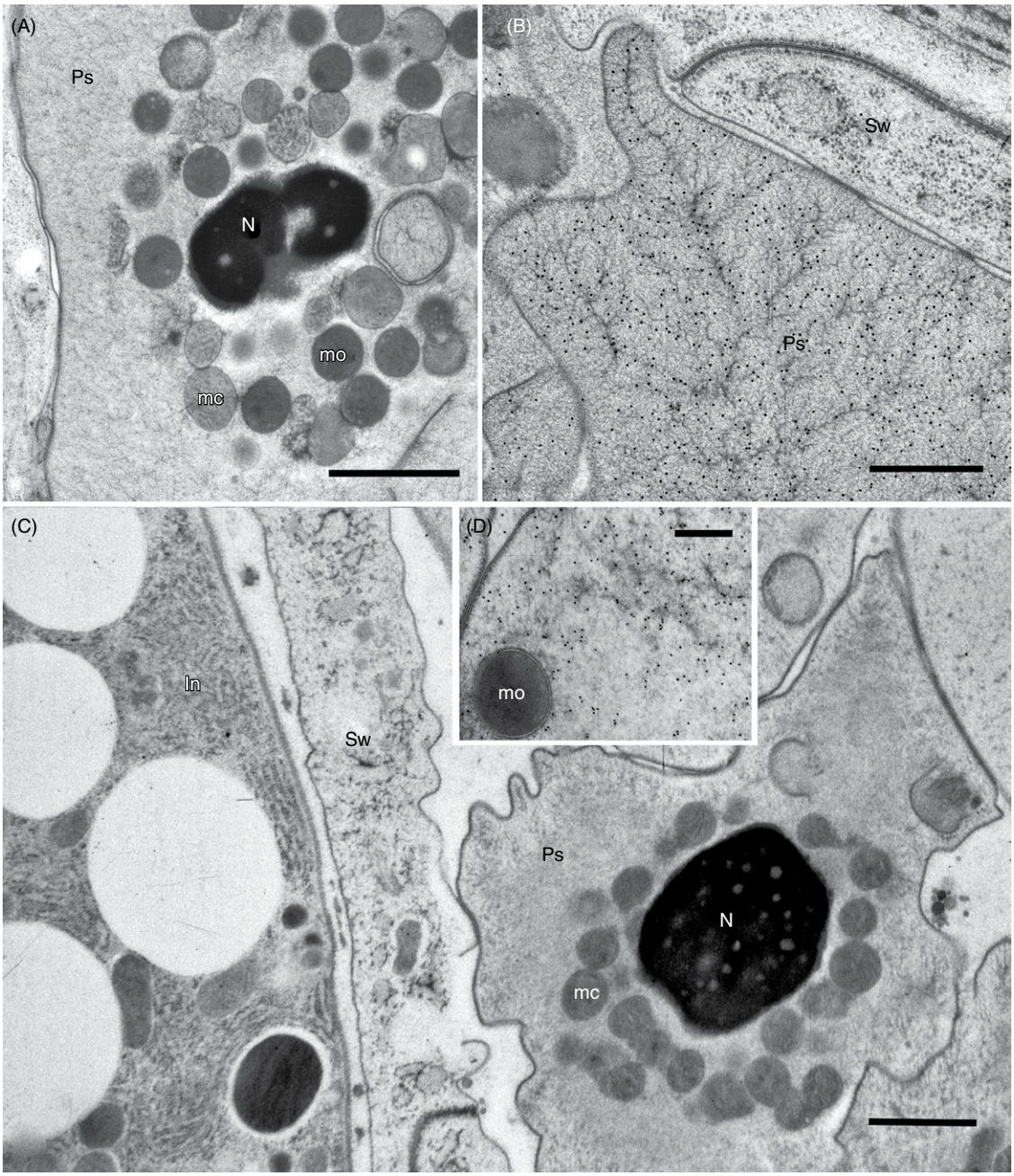

Fig. 8.5. *Acrobeles complexus* (Rhabditida, Cephalobidae); mature spermatozoa from spermatheca, TEM. Compare HPF/AFS **(A, B)** with SPRF/AFS **(C, D)** fixations (Tables 8.3, 8.4 and 8.5); all specimens embedded in Spurr epoxy resin; sections are double-stained with uranyl acetate and lead citrate. **(A)** Spermatozoon central part. **(C)** Sections shows spermatozoon in spermatheca, spermathecal wall and intestinal epithelium. **(B, D)** The sperm pseudopod at higher magnification; sections are labelled by antibody to major sperm protein (MSP) conjugated with colloidal gold (immunogold labelling, for details see Yushin *et al*., 2016). Note intense labelling over the filamentous content of the pseudopods. Abbreviations: In, intestine; mc, mitochondria; mo, membranous organelles; N, nucleus; Ps, pseudopod; Sw, spermathecal wall. Scale bars: A, C = 1 μm; B = 0.5 μm; D = 0.25 μm.

Table 8.3. Processing specimens for high-pressure freezing.

1. **Covering the membrane carriers.** Put one drop of 1% egg-lecithin in dried acetone on the carrier (use stereomicroscope). Let the acetone evaporate (a thin uniform layer of egg-lecithin remains to cover the carrier).
2. **Preparation of cryoprotectant**. For nematodes 20% BSA is advisable. For 1 ml solution of 20% BSA add 0.2 g of BSA powder into 1 ml of distilled water in an Eppendorf. Close the Eppendorf and centrifuge 5 min at 5000 rpm. Powder will be dissolved and the solution is ready to use. Store the surplus solution in the freezer.
3. **Sample loading steps.** Transfer the nematodes with a needle into a drop of cryoprotectant on a slide. Fill the membrane carrier with 20% BSA (use a binocular microscope and fill the cavity by forceps or use a micropipette). Transfer the nematodes from the slide into the copper carrier. Procedure needs practice, add at least three nematodes. Ensure that the carrier is completely filled with cryoprotectant (adjust if necessary). Close the membrane carrier and freeze in the HPF equipment using instruction manual.

Caution: Adhere to the safety guidelines regarding handling of liquid nitrogen! This remark applies to all subsequent cryomethods.

For nematodes, the most commonly used specimen holder is the copper membrane carrier that varies in diameter (1.5–6.0 mm) and depth (0.1 mm, 0.2 mm or 0.3 mm). The carriers must be covered by egg-lecithin before use in order to facilitate removal of specimens from the carrier after the FS procedure (Table 8.3).

The inner cavity of the specimen holder must be filled with a cryoprotectant to avoid extracellular ice crystal formation and to provide the best freezing rate. Air bubbles within the holder act as insulators and collapse during pressurization. The filler should be a substance that cannot penetrate the tissue, has low osmotic activity and high cryoprotective ability. A comprehensive list of various cryoprotectants can be found in Bert *et al*. (2016).

8.1.8 Self-pressurized rapid freezing

The new, fast, easy-to-use and low-cost cryofixation method called self-pressurized rapid freezing (SPRF) can effectively replace freezing with expensive and immobile commercial HPF devices (Leunissen and Yi, 2009; Grabenbauer *et al*., 2014; Claeys *et al*., 2017, 2019; Huebinger and Grabenbauer, 2018). So far, the method has been successfully used for cultured nematodes, which are available in sufficient quantity. The method employs plunge freezing of specimens in a sealed capillary copper tube into a cryogen such as liquid nitrogen or nitrogen slush, alternatively liquid propane or liquid ethane (Table 8.4, illustrated by Fig. 8.6). The preservation of these unprotected specimens is comparable to that achieved with conventional HPF in the presence of a cryoprotectant (Figs 8.3B, 8.5 and 8.7). SPRF uses the tendency for water inside the specimen container (a confined tightly closed metal tube) to expand upon cooling, thereby generating pressure intrinsically instead of using an external hydraulic system. The expansion of water and the hexagonal ice during cooling causes increased pressure inside the tube and thus supports high pressure cryofixation of the sample (Leunissen and Yi, 2009; Han *et al*., 2012). To prevent poor heat transfer and cryo damage, nitrogen slush (NS), a semi-solid form of nitrogen obtained after evacuation of liquid nitrogen, which has a lower temperature (–205°C to –210°C) than liquid nitrogen (–196°C) and avoids the Leidenfrost phenomenon, is used. By preventing the formation of a gas layer due to boiling, quenching in NS provides faster cooling rates that result in better cryopreservation. Detailed description of the method as used with *C. elegans* is given in several publications (e.g. Leunissen and Yi, 2009; Grabenbauer *et al*., 2014; Huebinger *et al*., 2016). The method was also tested with soil nematodes and unhatched cyst nematodes with the quality of results equivalent to samples prepared by HPF device (Figs 8.3B, 8.5, 8.7; see also Claeys *et al*., 2017, 2019).

Table 8.4. Processing specimens for self-pressurized rapid freezing.

A. The evacuation chamber and nitrogen slush preparation.

1. Use a homemade evacuation chamber (Claeys *et al*., 2017) or an ordinary desiccator (e.g. Kartell Labware, Vol. 4,35 l, 200 mm) fitted with a valve (Fig 8.6A, B) connecting the rotary pump (e.g. RV Oil Sealed Rotary Vane Vacuum Pump. Edwards, Sussex, UK) to create nitrogen slush (NS).
2. Place a Styrofoam tray (inner space about 10 × 7 × 5 cm; 2.5 cm thick wall (Fig 8.5A)) inside the evacuation chamber and fill with liquid nitrogen (LN), close desiccator (Fig 8.6B).
3. Switch on the rotary pump, upon evacuation the liquid nitrogen is solidified at –210°C within minutes. The solidification process is visible through the translucent cover.
4. Switch off the rotary pump; the NS starts melting and needs to be used within 5 min.

B. Preparation of capillary copper tubes and filling with nematodes.

1. Copper capillaries are commercially available with an outer diameter of ~0.7 mm, inner diameter of ~0.35 mm, ~15 mm length (Leica, Vienna, Austria) (Fig. 8.6C, D). Alternatively, aluminium or silver tubes can be used (Goodfellow GmbH, Bad Nauheim, Germany; Grabenbauer *et al*., 2014; Huebinger and Grabenbauer, 2018).
2. Pre-clean the capillary copper tubes by 10 min sonication in pure acetone with following 3 × 10 min rinsing in distilled water.
3. Wash off living nematodes from the culture plate with distilled water and collect in a glass dish.
4. Insert the copper tube into a disposable pipette tip; mount on a 0–20 µl micropipette (Fig. 8.6E).
5. Insert the open end of the capillary tube into the specimen suspension and draw nematodes into the copper tube (until nematodes are visible on the opposite side of the tip) (Fig. 8.6E).
6. Remove the filled copper tube (still mounted in the pipette tip) and seal the open end by clamping a length of 1.0–1.5 mm shut using pliers with flat jaws.
7. While pressing the pipette piston down, remove the copper tube from the micropipette and seal the remaining open end. Container (the sealed copper tube) with nematodes now is ready for freezing (Fig. 8.6D).

C. Cryoprocessing.

1. Create NS in the Styrofoam tray.
2. Aerate the evacuation chamber.
3. Hold the sealed copper tube horizontally in the middle with fine forceps and immerse quickly into the NS (Fig. 8.6F)
4. Drop the capillary as soon as it is immersed and keep the specimens at least 15 s in the cryogen.
5. Transfer the copper tube under LN to another Styrofoam tray filled with LN.
6. Cut out the centre part of the copper tube (ideally ~ 5 mm long) using pre-cooled commercially available capillary cutting pliers (degreased to prevent stiffening of grease at LN temperatures) or wire strip pliers.
7. Transfer the copper segments under LN into a cryovial that contains LN cooled substitution liquid.
8. Close the cryovials and start the FS procedure.

8.1.9 Freeze substitution

The specimens cryofixed using HPF or SPRF must be dehydrated by freeze substitution (FS), the process of dissolution of ice in a frozen specimen by an organic solvent (acetone, methanol) at low temperature (–90°C) and then warming over a period of hours or days to higher temperatures to be embedded in resin (Cavalier *et al*., 2008). This procedure may include fixation by OsO_4, glutaraldehyde and uranyl acetate, depending on the experiment. There are widely distributed commercial devices for automatic control of specimens processing during freeze substitution (Leica AFS and Leica AFS2). Detailed explanation of the process is available in several guides (Weimer, 2006; McDonald *et al*., 2007; Hall *et al*., 2012; McDonald, 2014b; Manning and Richmond, 2015; Serwas and Dammermann, 2015). Agitation modules have been developed to accelerate automated freeze substitution (Reipert *et al*., 2018).

Freeze substitution employs fixatives in dried acetone (or methanol) at various percentages depending on whether the fixation is intended for morphological or post-embedding immunocytochemistry. A list of various freeze-substitution cocktails used in different kinds of studies on nematodes have been summarized by Bert *et al*. (2016). The outline of freeze-substitution procedures based on versions that gave excellent results with soil and plant-parasitic nematodes is given in Lak *et al*. (2015) and Yushin *et al*. (2016); see also Table 8.5. Freeze-substitution procedure may be performed in each laboratory without sophisticated equipment using liquid nitrogen and/or dry ice in an insulated styrofoam box or using the rapid method of quick-freeze

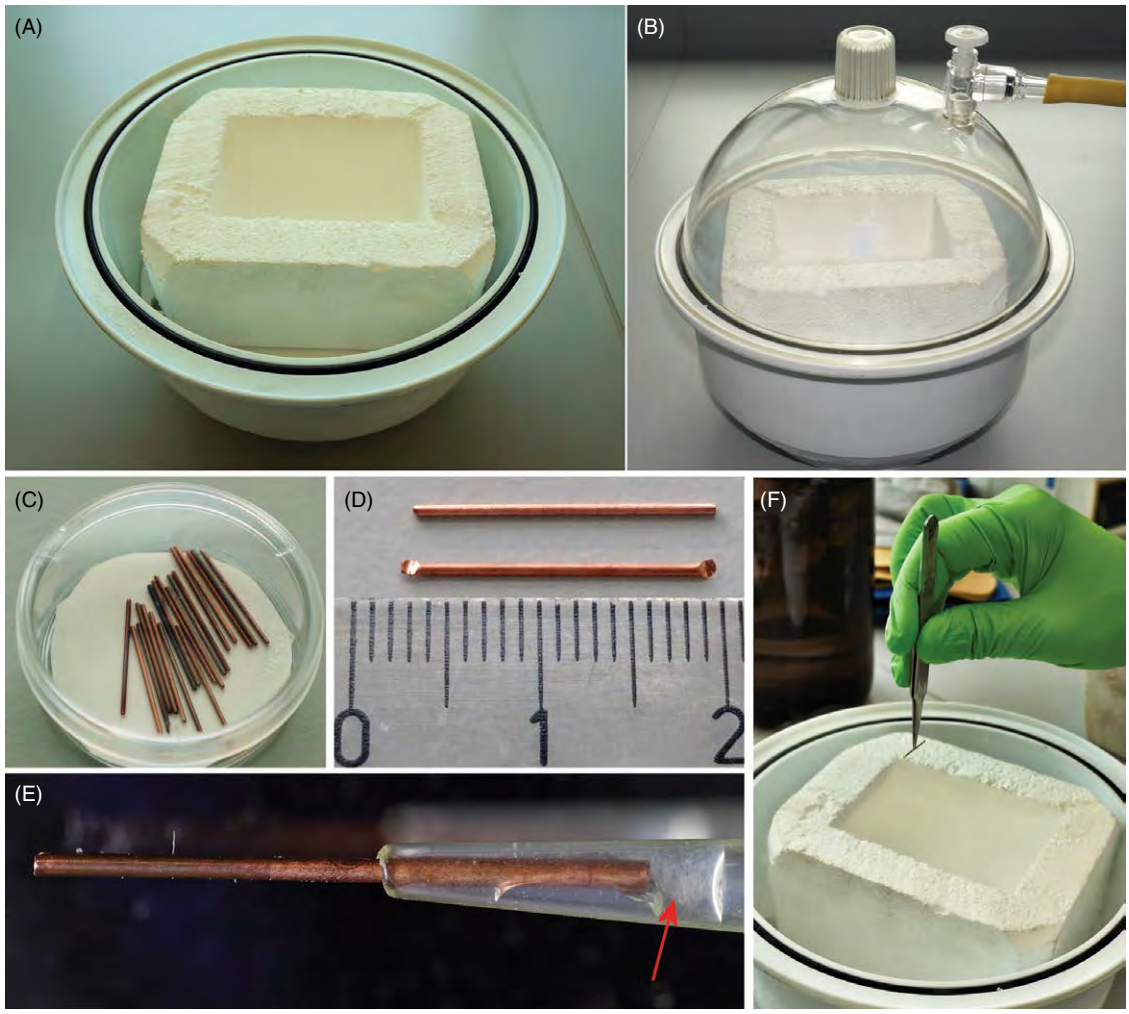

Fig 8.6. Self-pressurizing rapid freezing (SPRF), illustrations to Table 8.4. **(A)** The Styrofoam tray suitable for manipulations with liquid nitrogen and nitrogen slush (NS) positioned inside the evacuation chamber or vacuum desiccator. **(B)** Closed vacuum desiccator fitted with a valve joined by a hose to the vacuum pump. **(C)** Copper capillary tubes prepared before a series of SPRF procedures. **(D)** The clear copper capillary tube (upper) and container with nematodes, i.e. tube sealed at both ends by pliers (lower); scale bar = 2 cm. **(E)** The copper tube inserted as a continuation of the disposable pipette tip; the suction is halted when nematodes emerge in the pipette tip on the opposite side of the copper tube (visible inside the tip, *arrow*). **(F)** The sealed copper tube with nematodes inside held horizontally in the middle with fine forceps and rapidly submerged in the NS.

substitution that requires only ordinary laboratory tools (McDonald and Webb, 2011; McDonald, 2014a,b). It remains to be seen what the limitations of these simplified methods are for a wider range of specimens and in that sense for the time being comparisons with AFS results are recommended.

8.1.10 Preparation of sections for TEM observations

The basic result of tissue preparation for TEM is a collection of plastic blocks with specimens, which may be stored for many years before use. Standard operations of block trimming and subsequent section preparation

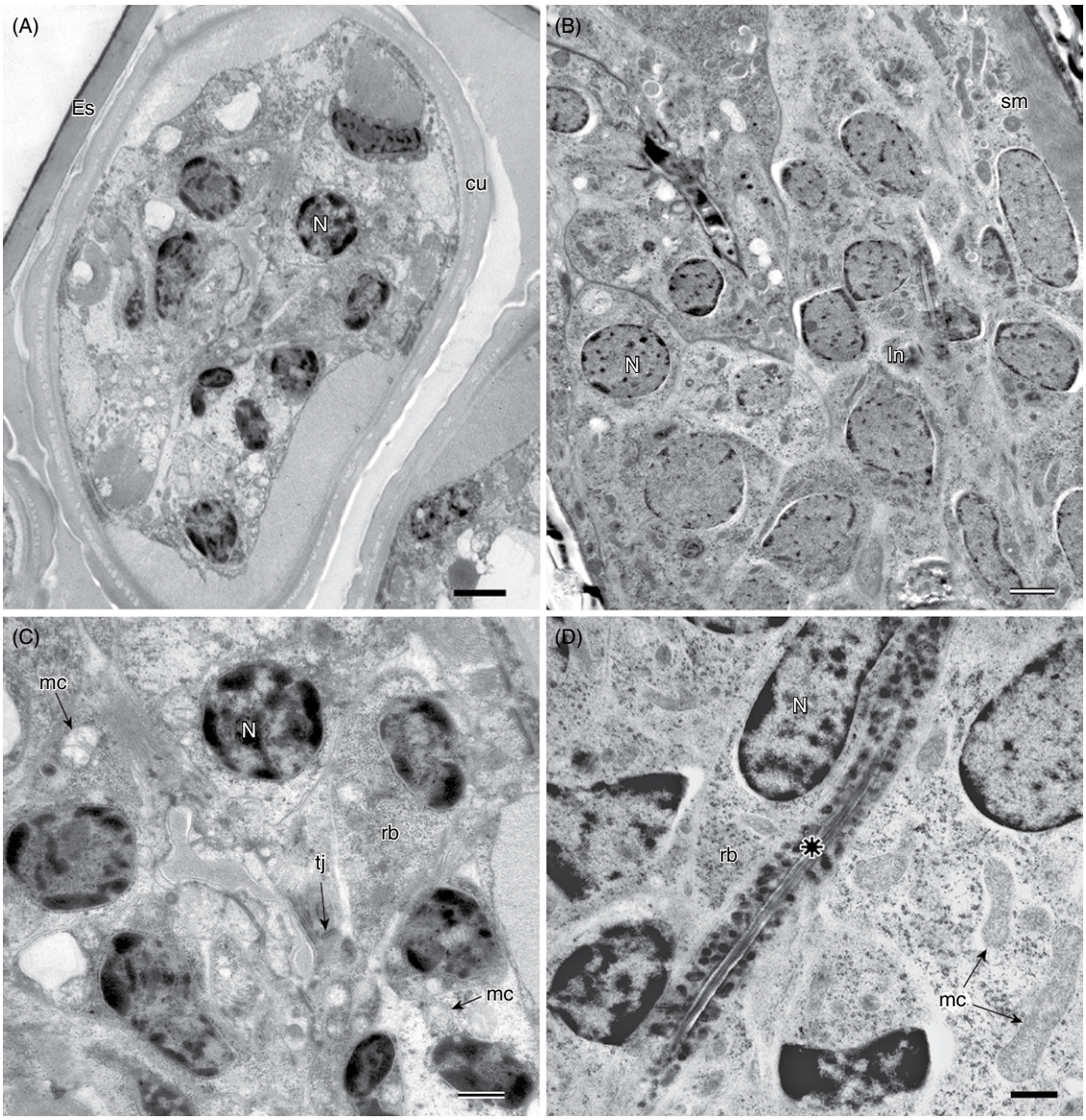

Fig. 8.7. Ultrastructure of the second-stage juvenile (J2) developing inside the egg of the cyst nematodes *Globodera rostochiensis* **(A, C)** and *Heterodera schachtii* **(B, D)**, TEM. SPRF/AFS (processed as in Tables 8.4 and 8.5). **(A)** Cross section through the J2 coiled inside the egg. **(B)** Longitudinal section of the J2. **(C)** Higher magnification from **(A)** showing details of cell structure. **(D)** Details of developing intestine; asterisk marks precursive lumen of the intestine. Abbreviations: cu, cuticle; Es, eggshell; In, intestine; mc, mitochondria; N, nucleus; rb, ribosomes; sm, somatic muscle cell; tj, tight junction. Scale bars: A, B = 1 µm; C, D = 0.5 µm. (From Claeys *et al.* (2019), courtesy of Brill, Leiden, The Netherlands.)

have been described in numerous guides, including modifications for processing nematodes (Bird, 1971; Shepherd and Clark, 1986; Carta, 1991; Hall, 1995; Serwas and Dammermann, 2015). The use of a diamond knife is strongly recommended for epoxy (and almost essential for acrylic) resins to make the cut not only

Table 8.5. AFS processing of specimens.

A. Substitution cocktail preparation (10 ml, solvent in dried acetone).

For morphology	For immunocytochemistry
9.4 ml dried acetone 0.5 ml GA, 10% solution in acetone 0.1 ml distilled water 100 mg OsO_4	9.4 ml dried acetone 0.5 ml GA, 10% solution in acetone 0.1 ml distilled water
Cocktail content: 1% OsO_4,[a] 1% H_2O,[b] 0.5% GA in dried acetone.	Cocktail content: 1% H_2O,[b] 0.5% GA[c] in dried acetone.

B. Freeze-substitution procedure.

1. **Transfer the membrane carrier** under LN from the HPF instrument (or the copper segment from the SPRF evacuation chamber or vacuum desiccator) into a cryovial (prefilled with substitution cocktail at liquid nitrogen temperature) in the FS device. Pre-cool all used tools, warming up of the carrier may be detrimental as long as frozen water has not been removed from the specimens.
2. **The FS device is programmed** for morphology as well as for immunocytochemistry as follows:
Incubation at –90°C (27 h)
Warm up to –60°C, slope 2°/h (15 h)
incubation at –60°C (12 h)
Warm up to –30°C, slope 2°/h (15 h)
incubation at –30°C (32 h)
Warm up to 4°C, slope 2°/h (17h)
3. **Remove the cryovials from the FS device** (place in a container with melting ice) and transfer to a fume hood.
4. **Replace the substitution cocktail** with pure dried acetone.
5. **Start infiltration and polymerization** with Spurr's resin (for morphology studies) or LR White (for immunocytochemistry).
Note: during the substitution and infiltration the contents will fall out of the specimen containers (carriers, copper tubes) on to the bottom of the cryovial. Any sample material remaining inside the capillary segments can be pushed out using an eyelash probe. In the case of the membrane carriers, sometimes a little help with a needle is necessary to extract the content.
6. **Polymerization.**

Using heat:	At low temperatures for immunocytochemistry (LR White, closed gelatine capsules):
Morphology (Spurr's resin and LR White): 65–70°C, 8 h (polyethylene or rubber moulds for Spurr, closed gelatine capsules for LR White) Immunocytochemistry (LR White): 37°C, 1 week (closed gelatine capsules).	By UV light in the FS device, use special tools and the following program for polymerization: Incubation at 0°C (24 h) Warm up to 20°C, slope 2°/h (10 h) Incubation at 20°C (24 h) Warm up to 37°C, slope 2°/h (8.5 h) Final incubation at 37°C (72 h).

[a] Concentration of OsO_4 can be reduced up to 0.1%.
[b] Water is used as an additive and may be omitted (Bert *et al*., 2016).
[c] Concentration of GA for immunocytochemistry may be reduced to 0% (De Henau *et al.*, 2015).

perfect, but also easy and fast. The procedure for serial sectioning has been detailed using *C. elegans* as a model (Hall, 1995; Hall *et al*., 2012; Serwas and Dammermann, 2015).

Thin sections must be stained (contrasted) before observations with an electron microscope. Standard two-step staining includes uranyl acetate followed by lead citrate solutions (Table 8.6). The formvar-coated single-slot grids with thin sections may be automatically stained if equipment such as Leica EM AC20 is available. Short series of grids may be stained easily using simple laboratory devices (Shepherd and Clark, 1986;

Table 8.6. Double staining of thin sections.

1. **Uranyl acetate (UA) stain preparation**: 1% UA in DW with 10% of ethanol.
2. **Lead citrate stain preparation**, 10 ml (based on Reynolds, 1963). Dissolve 133 mg of lead nitrate ($PbNO_3$) in 9.2 ml of DW. Add 175 mg of sodium citrate (trisodium salt dihydrate, $Na_3C_6H_5O_7$ $2H_2O$). Stir suspension for about 30 min. Add 0.8 ml of freshly prepared 1M NaOH and stir. Solution turns transparent immediately. Aliquot the stain into 1 ml tubes and store in a fridge until use.
3. **Uranyl acetate staining**: 20–30 min at 37°C. Protect from light.
4. **Lead citrate staining**: 2–4 min at room temperature. Protect from atmospheric CO_2.

Bozzolla and Russell, 1999; Hall *et al*., 2012; Serwas and Dammermann, 2015). The *en bloc* staining of specimens by uranyl acetate (Table 8.1) is usually enough for specimens embedded into Epon-like or acrylic resins. Alternatively, the sections may be stained by lead citrate only or may even be observed without staining. Spurr's resin sections must be stained by uranyl acetate up to 30 min at higher temperature with ethanol as an additive (Table 8.6). Double stain used for epoxy resins is effective also for tissues fixed without osmium tetroxide and embedded in acrylic resins (Fig. 8.1).

Double staining sometimes results in precipitation of chemicals. Overstaining and precipitation may be easily removed by 1 min washing of grids in 0.01 M NaOH solution, rinsing in distilled water and repetition of both stain procedures.

8.1.11 Transmission electron microscopy

Conventional TEM methods are well established after more than 70 years use in biology. Each newcomer needs to study general books on tissue and cell ultrastructure before the start of their own observations (Bozzolla and Russell, 1999). For nematologists, there are important books where ultrastructure of all nematode tissues and cell types has been analysed and illustrated (Bird, 1971; Bird and Bird, 1991; Wright, 1991; Malakhov, 1994). More detailed information on nematode ultrastructure may be found in atlases on *C. elegans* (Hall and Altun, 2008) and on the infective juveniles of the plant-parasitic nematode *Heterodera glycines* (Endo, 1998). Several review papers and book chapters are also available that focus on a specific subject, such as nematode cuticle, sense organs, nervous system and gametes (McLaren, 1976; Bird, 1984; White *et al*., 1986; Justine, 2002; Decraemer *et al*., 2003).

Immuno-electron microscopy, which combines sensitive protein detection with detailed information on the cell ultrastructure, is now well developed as immunogold labelling techniques for precise ultrastructural localization, distribution and quantification of macromolecules in cryofixed or chemically fixed specimens (Möbius, 2016). Each new scientific problem requires modifications of immunogold labelling protocols, starting from methods of fixation and choice of antibodies. However, general books about the techniques of IEM (e.g. Schwartzbach and Osafune, 2010) are available as well as reviews and research papers on the IEM methods specially developed in detail for study of *C. elegans* and other nematodes (Hall, 1995; Claeys *et al*., 2004, 2017; Hall *et al*., 2012; Weimer, 2006; Morphew, 2007; Bert *et al*., 2016; Yushin *et al*., 2016; Figs 8.2C,D and 8.5B,D).

Observations of separate sections sometimes are not sufficient for a comprehensive morphological analysis. Serial sections made with a diamond knife provide successive images that are useful for 3D reconstructions of organs, tissues, cells and organelles. The reconstruction procedures are now well established and computerized, and are described in many guides, including those specially dedicated to nematodes (Hall, 1995; Hall *et al*., 2012; Müller-Reichert *et al*., 2010; Mulcahy *et al*., 2018). Reconstruction from serial images needs only a conventional electron microscope and a computer with specialized software (Miranda *et al*., 2015). Nematodes, with their small size and clearly arranged tissues, are suitable objects for reconstruction

of gross morphology (Bumbarger *et al*., 2009; Ragsdale *et al*., 2011; Riebesell and Sommer, 2017). However, serial sections analysis is a labour-intensive method and its selection needs convincing reasons.

Modern computerized electron microscopes with reliable CCD cameras, powerful software and tomography capability are used for the creation and annotation of 3D tomograms of thin and semithin sections (Kuo, 2014; Ishikawa, 2016). Electron tomography acquires a series of projected images from different perspectives as the sample is rotated incrementally about an axis perpendicular to the viewing direction. A computer then combines these images into a three-dimensional model of the sample. Nematodes preserved by HPF/FS techniques are ideal specimens for electron tomography; however, high-resolution tomograms of sections at the level of molecular aggregates and organelles are more suitable for cell and developmental biology than for conventional nematology (Müller-Reichert *et al*., 2010; König *et al*., 2017). A review by Miranda *et al*. (2015) on different methods of studying 3D images in biology is recommended to make a choice depending on the objective of an investigation.

New approaches in TEM studies are developing rapidly due to modern cryomethods that became the basis of cryo-sectioning, electron tomography and 3D reconstruction. These new methods include ion beam scanning electron microscopy (FIB SEM), CLEM and *in situ* hybridization in electron microscopy (Kuo, 2014; Miranda *et al*., 2015; Ishikawa, 2016; Möbius, 2016). These modern methods to investigate cell and developmental biology are applicable to soil and plant nematodes and are comprehensively reviewed in several papers and chapters (Serwas and Dammermann 2015; Bert *et al*., 2016; König *et al*., 2017). New electron microscopy methods may be used in a variety of nematology studies, including comparative anatomy and embryology in systematic studies, functional morphology based on immunolocalization of cell proteins, characterization of vector viruses and symbiotic bacteria, host–parasite interactions, toxicology and environmental pollution, and nematicide targets and efficiency.

8.1.12 Concluding remark on TEM applications

The cryo-fixation by HPF as a starting point for several associated TEM preparation methods is likely soon to become the accepted standard in nematode fixation techniques (Bert *et al*., 2016). The SPRF method, as the low-cost alternative to HPF (Leunissen and Yi, 2009; Grabenbauer *et al*., 2014; Claeys *et al*., 2017, 2019; Huebinger and Grabenbauer, 2018), increases the availability of adequate cryo-fixation methods for nematology research.

However, cryo-fixation and related methods, being relatively expensive and/or complicated, do not replace the basic chemical procedures in TEM. For studies that do not require greatest preservation of cell components, high-resolution, high-quality results, or the immediate arrest of certain processes, conventional methods of TEM will provide answers to many questions in nematology (Bert *et al*., 2016). Methods of fast fixation, processing and embedding in acrylic and epoxy resins change the image of conventional TEM preparation from laborious lengthy procedures to straightforward, rapid techniques that may be started and finished inside a working day (Bozzolla and Russell, 1999; McDonald and Webb, 2011; McDonald, 2014a,b).

8.2 Scanning Electron Microscopy

Observations by means of the scanning electron microscope (SEM) are focused on the external features of a specimen and are widely used in nematode taxonomy as a routine method for description and illustration of new species (Fig. 8.8A–C). Internal structures of nematodes also may be investigated by fracturing or dissections of the specimens (Wergin, 1981; Eisenback, 1985; Shepherd and Clark, 1986; Adnet *et al*., 2013). To understand the basic SEM methods, chapters in various handbooks are recommended (e.g. Bozzolla and Russell, 1999; Fischer *et al*., 2012; Bozzolla, 2014; Allen and Goldberg, 2016). SEM methods have a long history in nematology and have been reviewed in a series of guides specifically dedicated to nematodes (Green *et al*., 1975; Wergin, 1981; Shepherd and Clark, 1986; Eisenback, 1985, 1986, 1991). Here the common reagents and procedures used to prepare nematode specimens for SEM observations are briefly outlined and references to basic techniques are included.

8.2.1 Preparation of nematodes for SEM

A scanning electron microscope produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about

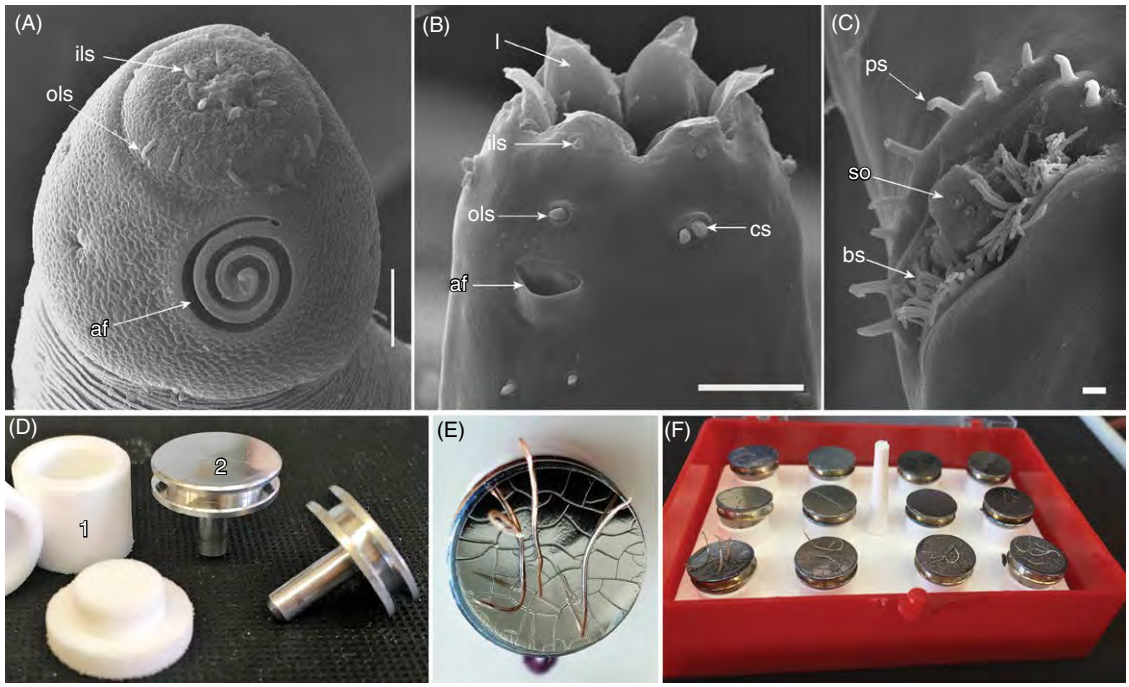

Fig. 8.8. Scanning electron microscopy (SEM). **(A–C)** SEM pictures of nematodes made for taxonomical descriptions. **(A)** *Zalonema kamchatkaensis,* head end, lateral view (from Fadeeva *et al*., 2016; reproduced with permission from copyright holder, Magnolia Press.). **(B, C)** *Adoncholaimus ussuriensis*: **(B)** head end, ventro-lateral view; **(C)** cloacal region, ventral view (from Mordukhovich *et al*., 2015). **(D)** Microporous specimen capsules for nematode dehydratation (1) and aluminum specimen mount stubs (2). **(E)** Nematodes mounted onto the specimen mount stub after sputter coating with chrome. **(F)** Storage holder containing specimen mount stubs with a series of coated nematodes. Abbreviations: af, amphidial fovea; bs, bands of cloacal setae; ils, inner labial sensilla; l, lip; ols, outer labial sensillum; ps, precloacal seta; sc, cephalic seta; so, supplementary organ. Scale bars: A, B = 10 µm; C = 2 µm.

the sample's surface topography and composition. The electron beam is generally scanned in a raster scan pattern, and the beam's position is combined with the detected signal to produce an image. Specimens can be observed in high vacuum, in low vacuum, in wet conditions (in environmental SEM), and at a wide range of cryogenic or elevated temperatures. The first steps for SEM preparation are similar to TEM methods: fixation in buffered aldehyde, post-fixation in osmium tetroxide and dehydrating in organic solvents. The following are specific procedures such as drying, mounting on a stub, coating with a heavy metal and observation with scanning electron microscope.

8.2.2 Fixation

Most plant-parasitic and soil nematodes are small and have to be handled through the various steps of processing enclosed in a container. Such containers should allow for a rapid exchange of fluids, minimize loss of specimens, reduce shock to tissues and restrict contamination (Eisenback, 1985, 1991). The most widely used and highly recommended chamber is easily made from two polyethylene BEEM capsules that are available from several suppliers (Eisenback, 1985). Several other handmade containers have been constructed and may be chosen depending on specimen character (Annells, 1985; Eisenback, 1985; Shepherd and Clark, 1986). Other holders or chambers to hold small nematodes during processing are also available through various microscopy suppliers (Fig. 8.8D).

The specimens are picked or pipetted in the container where most of a solution is withdrawn from the container to be replaced with the next solution. Prior to critical-point drying, the chamber is closed with a filter and cap (Eisenback, 1985).

Fixation is the first and most important step in preparing nematodes for SEM. Conventional fixation for TEM with aldehyde fixers and post-fixation by osmium tetroxide, as described in Table 8.1, may be used for SEM preparations. However, for regular taxonomic observations the first aldehyde fixation may be simplified. Nematodes fixed in freshly prepared 4% formaldehyde in phosphate buffered saline (pH 7) are suitable for SEM studies (Annells, 1985). Using formaldehyde to fix samples of recovered dead nematodes for identification and documentation is also widely used (Wergin, 1981; Shepherd and Clark, 1986).

After aldehyde fixation, nematodes should be washed several times in the buffer or in distilled water. Since the surface of the specimen is the region of interest it must be cleaned thoroughly prior to or after fixation. For this purpose, various mild detergents (e.g. diluted shampoo or dishwashing liquid), surfactants (e.g. 0.03% Agral solution or eye make-up remover) or sonication for 45 s several times may be used. However, sonication requires caution in order to avoid damaging the nematodes.

Post-fixation in 1% buffered or aqueous osmium tetroxide gives good results to prevent nematode shrinkage during the drying procedure. Post-fixing is beneficial since osmium tetroxide stains nematodes black, making them easier to see during subsequent processing, and increases their conductivity in the SEM (Eisenback, 1985; Shepherd and Clark, 1986). After osmium fixation, material should be washed in distilled water three to four times.

Before drying, the procedure of gradual dehydration, as for TEM, should be used to avoid osmotic shock and to ensure complete removal of water. Dehydrate nematodes in an ethanol series for 15–30 min each step (7, 15, 30, 50, 70, 90, 96 and 100% ethanol concentrations) finishing with three changes of absolute ethanol.

8.2.3 Drying

Drying of nematodes is necessary because of the physical requirements of the SEM. The surface tension of water drying from the specimens exerts tremendous pressures that cause severe distortions (Eisenback, 1985, 1986). Specimens may be dried in a variety of ways, depending on the nature of the specimen but critical-point drying is the ideal method to use (Shepherd and Clark, 1986).

8.2.3.1 Critical-point drying

Liquids have a 'critical point' where both vapour and liquid states coexist, allowing surface tension effects to be avoided if vapour is removed above this critical point (Allen and Goldberg, 2016). The critical point for CO_2 at 31.1°C at a pressure of 10.13 MPa is compatible for the method. The organic solvent (e.g. ethanol), in a critical-point drying device is replaced by liquid CO_2, converted to vapour and slowly and carefully vented. The specimens become dry and are ready for mounting and coating.

For nematodes, dehydration in alcohol must be improved by a series of mixtures of ethanol/acetone or ethanol/amyl acetate (3:1; 1:1; 1:3) finishing with three changes of 100% acetone or amyl acetate. Then, nematodes are transferred to a critical-point drier (Eisenback, 1985; Shepherd and Clark, 1986). This method is the best for SEM but requires specialized equipment that may be unavailable, so other ways of drying are given below and by Eisenback (1985).

8.2.3.2 Air drying

This is the least recommended method because it imposes a severe stress on the nematode tissues resulting in surface shrinkage and distortion. Only tissues that can withstand this stress such as stylets or cysts can be treated in this way (Eisenback, 1985; Shepherd and Clark, 1986).

The air drying using hexamethyldisilazane (HMDS) is a well-developed method that is successfully used for SEM studies of a variety of biological objects, including nematodes (Nation, 1983; Hochberg and Litvaitis, 2000; Sudhaus *et al*., 2011; Fischer *et al*., 2012; Lee and Chow, 2012; Koon *et al*., 2019). In many cases the results are comparable with critical-point drying and other elaborate methods.

The simplest method includes only transfer of specimens dehydrated in ethanol into HMDS for 5–10 min with subsequent air drying at room temperature (Nation, 1983; Hochberg and Litvaitis, 2000; Wolff, 2011; Bozzolla, 2014). The apparent advantages of the HMDS method are low cost, simplicity and rapid procedure (Lee and Chow, 2012). For stable representative results more elaborated methods of air (chemical) drying in HDMS or t-butyl alcohol (TBA) were developed and may be recommended as the alternative to critical-point drying (Koon *et al*., 2019).

8.2.3.3 Acetone drying

This quick and simple method is suitable in the case of robust nematodes or nematodes with a well-developed head skeleton (Shepherd and Clark, 1986). Fixed and washed nematodes are placed into a glass cavity block over acetone in a desiccator for 24 h at room temperature. The acetone replaces the water and, since it has low surface tension and is highly volatile, the air/water interface passes quickly through the specimen and leaves it dry and ready for mounting (Green *et al*., 1975; Shepherd and Clark, 1986).

8.2.3.4 Freeze drying

A freeze-drying machine may be used for specimens that would be damaged by the critical-point drying procedure. Freeze drying was introduced to dehydrate biological samples because it causes less shrinkage than with critical-point drying. In freeze drying, the specimen is quickly frozen in a cryogen, such as liquid nitrogen, and the drying is performed in a freeze-dryer through sublimation of ice. The sublimation avoids the high surface tension at the liquid water–air interface, which causes disruption of cell topography during phase transformation. Substitution of cellular water by ethanol may not be necessary but is recommended due to its cryoprotective effect (Lee and Chow, 2012). After fixation and washing, nematode samples are dehydrated through a graded alcohol series, transferred to a freeze-drier and kept frozen under vacuum until the ice has sublimed when the specimens are dried (Shepherd and Clark, 1986; Eisenback, 1991).

8.2.3.5 Freeze substitution

If freeze-substitution equipment is available, it may be used for SEM as well as for TEM preparations. Freeze substitution proved superior to the other techniques used, including chemical fixation, resulting in good surface preservation, little structural collapse and the retention of the coiled posture exhibited by some nematodes. The process of specimen preparation is analogues to TEM procedures with finish in pure dried acetone (Table 8.5). Handmade equipment and modes of operation for processing by freeze substitution have been developed especially for studies of nematodes by Wharton (1991). Freeze substitution without commercial devices has been developed for TEM (McDonald and Webb, 2011; McDonald, 2014a,b) and may be used in the same way for SEM preparations.

8.2.3.6 Resin infiltration

Resin infiltration methods are described in the literature and may be used for preparation of nematodes when special equipment is not available (Wergin, 1981; Shepherd and Clark, 1986), but final results depend on the expertise of the user and the methods seems not to be easy.

8.2.4 Glycerin embedded specimens

In taxonomy sometimes the only available specimens will have already been processed to glycerin. With nematodes there are two ways to proceed (Eisenback, 1985, 1991; Shepherd and Clark, 1986).

One way is to place glycerin-infiltrated specimens on a piece of filter paper so the excess of glycerin is drained. Glycerin impregnated nematodes are flaccid and to ensure that they are correctly positioned for viewing they must be supported by a piece of fine wire stuck to the stub. Also, any excess of glycerin must be removed or it will obscure the specimen and contaminate the microscope column. The specimens are then coated (see Section 8.2.7) and viewed at relatively low accelerating voltage of 3 to 10 kV. Specimens are sensitive to beam damage and cannot be viewed for long periods at high magnifications.

Another way is to rehydrate nematodes in distilled water for at least 1 h at room temperature and then dehydrate them in ethanol series following standard procedures (Eisenback, 1985, 1986, 1991; see also Section 8.2.3.1 Critical-point drying).

8.2.5 Cryofracture

Morphological studies of nematodes by scanning electron microscopy are generally limited to the external topography of organisms. To see internal structures of nematodes it is necessary to cut the material. Cutting fresh material causes compression of the tissues at the point where the cut is made and they appear distorted. To avoid this, material can be frozen rapidly using liquid nitrogen and then fractured. Several methods of cryofracture have been developed specially for nematodes (Wergin, 1981; Eisenback, 1985; Shepherd and Clark, 1986). To obtain the optimal results it is recommended that critical-point drying is performed immediately after fracture (Adnet *et al*., 2013).

8.2.6 Mounting and coating

After specimens have been fixed and dried, they are mounted on an aluminium planchet, or specimen stub (Fig. 8.8D). A piece of double-coated adhesive tape or conductive carbon adhesive tabs can be attached on top of the stub and a short length of hair is placed on the tape. The nematodes are transferred from the specimen container to the stub with a preparation needle and are propped up against a hair to ensure adequate viewing angles (Eisenback, 1985).

Mounted specimens are normally coated with a thin layer of heavy metal, which serves as a source of secondary electrons and electrically grounds the specimen to prevent the build up of a high voltage static charge from the electron beam (Fig. 8.8E). The metal (carbon, chrome, silver, gold, platinum, gold/palladium alloy, etc.) can be applied to the surface either by evaporation or sputtering in a specialized device. Sputter coating is the most commonly used method because it is fast, reliable and the apparatus is relatively affordable. Vacuum evaporation coating is sometimes used when a sputter coater is not available or if higher resolution coating is needed. The stubs with coated specimens must be stored in plastic containers protected from dust (Fig. 8.8F).

The basic processes of specimen preparation for SEM described above are suitable for regular taxonomy and morphology of soil and plant-parasitic nematodes. For SEM use in cell, developmental and molecular biology of nematodes numerous modern methods have been developed during the last two decades and are described elsewhere (Allen and Goldberg, 2016).

8.3 Acknowledgements

V.V. Yushin and J.K. Zograf were supported in part by the Russian Science Foundation for the Far Eastern Federal University, project no. 14-50-00034 (studies of chemically fixed specimens), RFBR grant # 17-04-00719A and Foundation for Scientific Research Flanders grant FWOKAN2013001201 (studies of immunogold labelling in Ghent University, manuscript preparation). M. Claeys and J. Leunissen were supported by the TEM-expertise centre (Life Sciences) of Ghent University, Belgium. The Ghent University Russia Platform facilitate and stimulate cooperation between Russia and Belgium.

The authors are grateful to D.V. Fomin (Far East Centre of Electron Microscopy, NSCMB, Vladivostok, Russia) and Jef Claeys (Ghent University, Belgium) for technical assistance. The monoclonal MSP antibody developed by David Greenstein was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biology, Iowa City, Iowa 52242, USA.

8.4 References

Adnet, F.A. de O., Goncalves, J.P., de Souza, W. and Attias, M. (2013) A simple and efficient method to observe internal structures of helminths by scanning electron microscopy. *Microscopy and Microanalysis* 19, 1470–1474. DOI: 10.1017/S1431927613013706

Allen, T.D. and Goldberg, M.W. (2016) Scanning electron microscopy in cell biology. In: Bradshaw, R.A. and Stahl, P.D. (eds) *Encyclopedia of Cell Biology*. Elsevier, Amsterdam, The Netherlands, pp. 14–21. DOI: 10.1016/B978-0-12-394447-4.20005-9

Annells, C.M. (1985) Preparation of nematodes for scanning electron microscopy. *Nematologia Mediterranea* 13, 97–101.
Baranowski, Ł., Kurek, W., Lichocka, M. and Sobczak, M. (2018) A cryotechnique-based method for low abundance protein immunolocalization in tomato (*Solanum lycopersicum*) roots infected with a nematode, *Globodera rostochiensis*. *Micron* 108, 24–30. DOI: 10.1016/j.micron.2018.02.003
Basyoni, M.M.A. and Rizk, E.M.A. (2016) Nematodes ultrastructure: complex systems and processes. *Journal of Parasitic Diseases* 40, 1130–1140. DOI: 10.1007/s12639-015-0707-8
Bert, W., Slos D., Leroux, O. and Claeys, M. (2016) Cryo-fixation and associated developments in transmission electron microscopy: a cool future for nematology. *Nematology* 18, 1–14. DOI: 10.1163/15685411-00002943
Bird, A.F. (1971) *The Structure of Nematodes.* Academic Press, New York, New York, USA.
Bird, A.F. (1984) Nematoda. In: Bereiter-Hahn, J., Matoltsy, A.G. and Richards, K.S. (eds) *Biology of the Integument. Vol. 1. Invertebrates*. Springer, Berlin, Germany, pp. 212–233.
Bird, A.F. and Bird, J. (1991) *The Structure of Nematodes*, 2nd edn. Academic Press, New York, New York, USA.
Bozzolla, J.J. (2014) Conventional Specimen Preparation Techniques for Scanning Electron Microscopy of Biological Specimens. In: Kuo, J. (ed.) *Electron Microscopy: Methods and Protocols. Methods in Molecular Biology* 1117, 133–150. DOI: 10.1007/978-1-62703-776-1_7
Bozzolla, J.J. and Russell, L.D. (1999) *Electron Microscopy: Principles and Techniques for Biologists*, 2nd edn. Jones and Bartlett, Boston, Massachusetts, USA.
Bumbarger, D.J., Crum, J., Ellisman, M.H. and Baldwin, J.G. (2006) Three-dimensional reconstruction of the nose epidermal cells in the microbial feeding nematode, *Acrobeles complexus* (Nematoda: Rhabditida). *Journal of Morphology* 267, 1257–1272. DOI: 10.1002/jmor.10456
Bumbarger, D.J., Wijeratne, S., Carter, C., Crum, J., Ellisman, M.H. and Baldwin, J.G. (2009) Three-dimensional reconstruction of the amphid sensilla in the microbial feeding nematode, *Acrobeles complexus* (Nematoda: Rhabditida). *Journal of Comparative Neurology* 512, 271–281. DOI: 10.1002/cne.21882
Carta, L.K. (1991) Preparation of nematodes for transmission electron microscopy. In: Nickle, W.R. (ed.) *Manual of Agricultural Nematology*. Marcel Dekker, New York, New York, USA, pp. 97–106.
Cavalier, A., Spehner, D. and Humbel, B. (2008) *Handbook of Cryo-Preparation Methods for Electron Microscopy.* CRC Press Inc., Boca Raton, Florida, USA.
Claeys, M., Vanhecke, D., Couvreur, M., Tytgat, T., Coomans, A. and Borgonie, G. (2004) High-pressure freezing and freeze substitution of gravid *Caenorhabditis elegans* (Nematode: Rhabditida) for transmission electron microscopy. *Nematology* 6, 319–327. DOI: 10.1163/1568541042360564
Claeys, M., Yushin, V.V., Leunissen, J.L.M., Claeys, J. and Bert, W. (2017) Self-pressurised rapid freezing (SPRF): an easy-to-use and low-cost alternative cryo-fixation method for nematodes. *Nematology* 19, 871–881. DOI: 10.1163/15685411-00003093
Claeys, M., Handayani, N.D., Yushin, V.V., Prabowo, L., Dikin, A., Helder, J. and Bert, W. (2019) Self-pressurised rapid freezing (SPRF): a time efficient and affordable cryo-fixation method for ultrastructural observations on unhatched cyst nematodes. *Nematology* 21, 101–110. DOI: 10.1163/15685411-00003287
Decraemer, W., Karanastasi, E., Brown, D. and Backeljau, T. (2003) Review of the ultrastructure of the nematode body cuticle and its phylogenetic interpretation. *Biological Reviews* 78, 465–510. DOI: 10.1017/S1464793102006115
De Henau, S., Tilleman, L., Vangheel, M. *et al.* (2015) A redox signalling globin is essential for reproduction in *Caenorhabditis elegans*. *Nature Communications* 6, 8782. DOI: 10.1038/ncomms9782
Eisenback, J. (1985) Techniques for preparing nematodes for scanning electron microscopy. In: Barker, K.R., Carter, C.C. and Sasser, J.N. (eds) *An Advanced Treatise on* Meloidogyne*. Vol. 2 Methodology.* North Carolina State University Graphics, North Carolina State University, Raleigh, North Carolina, USA, pp. 79–105.
Eisenback, J. (1986) A comparison of techniques useful for preparing nematodes for scanning electron microscopy. *Journal of Nematology* 18, 479–487.
Eisenback, J.D. (1991) Preparation of nematodes for scanning electron microscopy. In: Nickle, W.R. (ed.) *Manual of Agricultural Nematology*. Marcel Dekker, New York, New York, USA, pp. 87–96.
Endo, B.Y. (1998) *Atlas on ultrastructure of infective juveniles of the soybean cyst nematode*, Heterodera glycines. Agriculture Handbook No 711. US Department of Agriculture, Washington DC, USA.
Fadeeva, N., Mordukhovich, V. and Zograf, J. (2016) Free-living marine nematodes of *Desmodorella* and *Zalonema* (Nematoda: Desmodoridae) with description of two new species from the deep sea of the North Western Pacific. *Zootaxa.* 4175, 501–520. DOI: 10.11646/zootaxa.4175.6.1
Fischer, E.R., Hansen, B.T., Nair, V., Hoyt, F.H. and Dorward, D.W. (2012) Scanning Electron Microscopy. *Current Protocols in Microbiology* 25, 2B.2.1–2B.2.47 DOI: 10.1002/9780471729259.mc02b02s25
Giblin-Davis, R.M., Kanzaki, N., De Ley, P., Williams, D.S., Schierenberg, E., Ragsdale, E., Yongsan, Z.Y. and Center, B.J. (2010) Ultrastructure and life history of *Myolaimus byersi* n. sp. (Myolaimina: Myolaimidae), a phoretic associate of the

crane fly, *Limonia schwarzi* (Alexander) (Limoniidae), in Florida. *Nematology* 12, 519–542. DOI: 10.1163/138855409X12519673803912
Glauert, A.M. and Lewis, P.R. (1998) *Biological Specimen Preparation for Transmission Electron Microscopy.* Portland Press. London, UK.
Grabenbauer, M., Han, H.-M. and Huebinger, J. (2014) Cryo-fixation by self-pressurized rapid freezing. *Methods in Molecular Biology* 1117, 173–191. DOI: 10.1007/978-1-62703-776-1_9
Green, C.D., Stone, A.R., Turner, R.H. and Clark, S.A. (1975) Preparation of nematodes for scanning electron microscopy. *Journal of Microscopy* 103, 89–99. DOI: 10.1111/j.1365-2818.1975.tb04540.x
Hajibagheri, M.A.N. (1999) *Electron Microscopy Methods and Protocols. Methods in Molecular Biology*, Vol. 117. Springer Science and Business Media, Berlin, Germany. DOI: 10.1385/1592592015
Hall, D.H. (1995) Electron microscopy and three-dimensional image reconstruction. *Methods in Cell Biology* 48, 395–436. DOI: 10.1016/S0091-679X(08)61397-7
Hall, D.H. and Altun, Z.F. (2008) C. elegans *Atlas*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.
Hall, D.H. and Rice, W.J. (2015) Electron tomography methods for *C. elegans.* In: Biron, D. and Haspel, G. (eds) C. elegans: *Methods and Applications, Methods in Molecular Biology*, Vol. 1327. Springer Science and Business Media, Berlin, Germany, pp. 141–158. DOI: 10.1007/978-1-4939-2842-2_11
Hall, D.H., Hartwieg, E. and Nguyen, K.C.Q. (2012) Modern electron microscopy methods for *C. elegans*. In: Rothman, J. (ed.) *Methods in Cell Biology*. Academic Press, New York, USA, pp. 93–149. DOI: 10.1016/B978-0-12-394620-1.00004-7
Han, H.-M., Huebinger, J. and Grabenbauer, M. (2012) Self-pressurized rapid freezing (SPRF) as a simple fixation method for cryo-electron microscopy of vitreous sections. *Journal of Structural Biology* 178, 84–87. DOI: 10.1016/j.jsb.2012.04.001
Han, Z., Thapa, S., Reuter-Carlson, U., Reed, H., Gates, M., Lambert, K.N. and Schroeder, N.E. (2018) Immobility in the sedentary plant-parasitic nematode *H. glycines* is associated with remodeling of neuromuscular tissue. *PLoS Pathology* 14, e1007198. DOI: 10.1371/journal.ppat.1007198
Hayat, MA. (2000) *Principles and Techniques of Electron Microscopy: Biological Applications*. 4th edn. Cambridge University Press, Cambridge, UK.
Hochberg, R. and Litvaitis, M.K. (2000) Hexamethyldisilazane for Scanning Electron Microscopy of Gastrotricha. *Biotechnic & Histochemistry* 75, 41–44. DOI:10.3109/10520290009047984
Huebinger, J. and Grabenbauer, M. (2018) Self-pressurized rapid freezing as cryo-fixation method for electron microscopy and cryopreservation of living cells. *Current Protocols in Cell Biology* 79, e47. DOI: 10.1002/cpcb.47
Huebinger, J., Han, H.-M. and Grabenbauer, M. (2016) Reversible cryopreservation of living cells using an electron microscopy cryo-fixation method. *PLoS ONE* 11, e0164270. DOI: 10.1371/journal.pone.0164270
Hurbain, I. and Sachse, M. (2011) The future is cold: cryo-preparation methods for transmission electron microscopy of cells. *Biology of the Cell* 103, 405–420. DOI: 10.1042/BC20110015
Ishikawa, T. (2016) Electron Tomography. In: Bradshaw, R.A. and Stahl, P.D. (eds) *Encyclopedia of Cell Biology*. Elsevier, Amsterdam, The Netherlands, pp. 22–31. DOI:10.1016/B978-0-12-394447-4.20006-0
Jones, J.T. and ap Gwynn, I.A. (1991) A method for rapid fixation and dehydration of nematode tissue for transmission electron microscopy. *Journal of Microscopy* 164, 43–51. DOI: 10.1111/j.1365-2818.1991.tb03190.x
Justine, J-L. (2002) Male and female gametes and fertilization. In: Lee, D.L. (ed.) *The Biology of Nematodes*. Taylor and Francis, London, UK, pp. 73–119.
Kaech, A. and Ziegler, A. (2014) High-pressure freezing: Current state and future prospects. *Electron Microscopy: Methods and Protocols. Methods in Molecular Biology*, *Vol. 1117*. Springer Science Business Media, New York, New York, USA. DOI: 10.1007/978-1-62703-776-1_8
Karahara, I. and Kang, B.H. (2014) High-pressure freezing and low-temperature processing of plant tissue samples for electron microscopy. In: Zarsky, V. and Cvrckova, F. (Eds) *Plant Cell Morphogenesis: Methods and Protocols*. *Methods in Molecular Biology 1080*, pp. 147–157. DOI: 10.1007/978-1-62703-643-6_12
Kolotuev, I., Bumbarger, D.J., Labouesse, M. and Schwab, Y. (2012) Targeted ultramicrotomy: a valuable tool for correlated light and electron microscopy of small model organisms. *Methods Cell Biology* 111, 203–222. DOI: 10.1016/B978-0-12-416026-2.00011-X
König, J., Borrego-Pinto, J., Streichert, D., Munzig, M., Lenart, P. and Müller-Reichert, T. (2017) Analysis of cytokinesis by electron microscopy. *Methods in Cell Biology* Vol. 137, 225–238. DOI: 10.1016/bs.mcb.2016.03.029
Koon, M.A., Almohammed Ali, K., Speaker, R.M., McGrath, J.P., Linton, E.W. and Steinhilb, M.L. (2019) Preparation of prokaryotic and eukaryotic organisms using chemical drying for morphological analysis in scanning electron microscopy (SEM). *Journal of Visualized Experiments* 143, e58761. DOI: 10.3791/58761
Kuo, J. (2014) *Electron microscopy: Methods and Protocols*, 3rd edn. *Methods in Molecular Biology, Vol. 1117*. Springer Science Business Media, New York, New York, USA. DOI: 10.1007/978-1-62703-776-1
Lak, B., Yushin, V.V., Slos, D., Claeys, M., Decraemer, W. and Bert, W. (2015) High-pressure freezing and freeze-substitution fixation reveal the ultrastructure of immature and mature spermatozoa of the plant-parasitic nematode *Trichodorus similis* (Nematoda; Triplonchida; Trichodoridae). *Micron* 77, 25–31. DOI: 10.1016/j.micron.2015.05.012

Lee, J.T.Y. and Chow, L.K. (2012) SEM preparation for cells in 3D scaffolds by freeze-drying and HMDS. *Scanning* 34, 12–25. DOI: 10.1002/sca.20271
Leunissen, J.L.M. and Yi, H. (2009) Self-pressurized rapid freezing (SPRF): a novel cryofixation method for specimen preparation in electron microscopy. *Journal of Microscopy* 235, 25–35. DOI: 10.1111/j.1365-2818.2009.03178.x
Li, X., Ji, G., Chen, X., Ding, W., Sun, L., Xu, W., Han, H. and Sun, F. (2017) Large scale three-dimensional reconstruction of an entire *Caenorhabditis elegans* larva using AutoCUTS-SEM. *Journal of Structural Biology* 200, 87–96. DOI:10.1016/j.jsb.2017.09.010
Malakhov, V.V. (1994) *Nematodes: Structure, Development, Classification, and Phylogeny*. Smithsonian Institution Press, Washington DC, USA.
Manning, M. and Richmond, J. (2015) High-pressure freeze and freeze substitution electron microscopy in *C. elegans*. In: Biron, D. and Haspel, G (eds) C. elegans: *Methods and Applications. Methods in Molecular Biology, Vol. 1327*. Springer Science and Business Media, Berlin, Germany, pp. 121–140. DOI: 10.1007/978-1-4939-2842-2_10
McDonald, K.L. (1999) High pressure freezing for preservation of high resolution fine structure and antigenicity for immunolabeling. In: Hajibagheri, M.A.N. (ed.) *Electron Microscopy Methods and Protocols. Methods in Molecular Biology* Vol. 117. Humana Press, Totowa, New Jersey, USA, pp. 77–97. DOI: 10.1385/1-59259-201-5:77
McDonald, K. (2007) Cryopreparation methods for electron microscopy of selected model systems In: McIntosh, J.R. (ed.) *Cellular Electron Microscopy. Methods in Cell Biology, Vol. 79.* Academic Press, New York, New York, USA, pp. 23–56. DOI: 10.1016/S0091-679X(06)79002-1
McDonald, K.L. (2014a) Rapid embedding methods into epoxy and LR White resins for morphological and immunological analysis of cryofixed biological specimens. *Microscopy and Microanalysis* 20, 152–163. DOI: 10.1017/S1431927613013846
McDonald, K.L. (2014b) Out with the old and in with the new: rapid specimen preparation procedures for electron microscopy of sectioned biological material. *Protoplasma* 251, 429–448. DOI: 10.1007/s00709-013-0575-y
McDonald, K.L. and Webb, R.I. (2011) Freeze substitution in 3 hours or less. *Journal of Microscopy* 243, 227–233. DOI: 10.1111/j.1365-2818.2011.03526.x
McDonald, K.L., Morphew, M., Verkade, P. and Müller-Reichert, T. (2007) Recent advances in high pressure freezing: Equipment and specimen loading methods. In: Kuo, J. (ed.) *Electron Microscopy Methods and Protocols* 2nd edn. *Methods in Molecular Biology Vol. 369*, pp. 143–173. DOI: 10.1007/978-1-59745-294-6_8
McLaren, D.J. (1976) Nematode sense organs. *Advances in Parasitology* 14, 195–265. DOI: 10.1016/S0065-308X(08)60515-1
Mielanczyk, L., Matysiak, N., Michalski, M., Buldak, R. and Wojnicz, R. (2014) Closer to the native state. Critical evaluation of cryo-techniques for Transmission Electron Microscopy: preparation of biological samples. *Folia Histochemica et Cytobiologica* 52, 1–17. DOI: 10.5603/FHC.2014.0001
Miranda, K., Wendell, G.-D., Attias, M., De Souza, W. and Ramos, I. (2015) Three-dimensional reconstruction by electron microscopy in the life sciences: An introduction for cell and tissue biologists. *Molecular Reproduction and Development* 82, 530–547. DOI: 10.1002/mrd.22455
Möbius, W. (2016) Immunoelectron microscopy: High-resolution immunocytochemistry. In: Bradshaw, R.A. and Stahl, P.D. (eds) *Encyclopedia of Cell Biology*. Elsevier, Amsterdam, The Netherlands, pp. 32–43. DOI: 10.1016/B978-0-12-394447-4.20007-2
Mordukhovich, V., Atopkin, D., Fadeeva, N., Yagodina, V. and Zograf J. (2015) *Admirandus multicavus* and *Adoncholaimus ussuriensis* sp. n. (Nematoda: Enoplida: Oncholaimidae) from the Sea of Japan. *Nematology* 17, 1229–1244. DOI: 10.1163/15685411-00002937
Morphew, M.K. (2007) 3D Immunolocalization with plastic sections. *Methods in Cell Biology* 79, 493–513. DOI: 10.1016/S0091-679X(06)79019-7
Mulcahy, B., Witvliet, D., Holmyard, D., Mitchell, J., Chisholm, A.D., Samuel, A.D.T. and Zhen, M. (2018) A pipeline for volume electron microscopy of the *Caenorhabditis elegans* nervous system. *Frontiers in Neural Circuits* 12, 94. DOI: 10.3389/fncir.2018.00094
Müller-Reichert, T.M., Srayko, M., Hyman, A., O'Tool, E. and McDonald, K.L. (2007) Correlative light and electron microscopy of early *Caenorhabditis elegans* embryos in mitosis. *Methods in Cell Biology* 79, 101–119. DOI: 10.1016/S0091-679X(06)79004-5
Müller-Reichert, T.M., Mäntler, J., Srayko, M. and O'Tool, E. (2008) Electron microscopy of the early *Caenorhabditis elegans* embryo. *Journal of Microscopy* 230, 297–307. DOI: 10.1111/j.1365-2818.2008.01985.x
Müller-Reichert, T.M., Mancuso, J., Lich, B. and McDonald, K. (2010) Three-dimensional reconstruction methods for *Caenorhabditis elegans* ultrastructure. In: Müller-Reichert, T. (ed.) *Electron Microscopy of Model Systems*. *Methods in Cell Biology* 96, 331–361. DOI: 10.1016/S0091-679X(10)96015-9
Nation, J.L. (1983) A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. *Stain Technology* 58, 347–351. DOI: 10.3109/10520298309066811
Newman, G.R. and Hobot, J.A. (1999) Resins for combined light and electron microscopy: A Half Century of Development. *Histochemical Journal* 31, 495–505. DOI: 10.1023/a:1003850921869

Ragsdale, E.J., Ngo, P.T., Crum, J., Ellisman, M.H. and Baldwin, J.G. (2011) Reconstruction of the pharyngeal corpus of *Aphelenchus avenae* (Nematoda: Tylenchomorpha), with implications for phylogenetic congruence. *Zoological Journal of the Linnean Society* 161, 1–30. DOI: 10.1111/j.1096-3642.2009.00632.x

Reipert, S., Goldammer, H., Richardson, C., Goldberg, M.W., Hawkins, T.J., Hollergschwandtner, E., Kaufmann, W.A., Antreich, S. and Stierhof, Y-D. (2018) Agitation modules: Fexible means to accelerate automated freeze substitution. *Journal of Histochemistry and Cytochemistry* 6, 903–921. DOI: 10.1369/0022155418786698

Reynolds, E.S. (1963) Use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* 17, 208–212. DOI: 10.1083/jcb.17.1.208

Riebesell, M. and Sommer, R.J. (2017) Three-dimensional reconstruction of the pharyngeal gland cells in the predatory nematode *Pristionchus pacificus*. *Journal of Morphology* 278, 1656–1666. DOI: 10.1002/jmor.20739

Rostaing, P., Weimer, R.M., Jorgensen, E.M., Triller, A. and Bessereau, J.L. (2004) Preservation of immunoreactivity and fine structure of adult *C. elegans* tissues using high-pressure freezing. *Journal of Histochemistry and Cytochemistry* 52, 1–12. DOI: 10.1177/002215540405200101

Schwartzbach, S.D. and Osafune, T. (2010) Immunoelectron microscopy, methods and protocols. *Methods in Molecular Biology, Vol. 657*. Springer, Berlin, Germany. DOI: 10.1007/978-1-60761-783-9

Serwas, D. and Dammermann, A. (2015) Ultrastructural analysis of *Caenorhabditis elegans* cilia. *Methods in Cell Biology* 129, 341–367. DOI: 10.1016/bs.mcb.2015.03.014

Shepherd, A.M. and Clark, S.A. (1986) Preparation of nematodes for electron microscopy. In: Southey, J.F. (ed.) *Laboratory Methods for Work with Plant and Soil Nematodes*. Her Majesty's Stationery Office, London, UK, pp. 121–131.

Spurr, A.R. (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* 26, 31–43. DOI: 10.1016/S0022-5320(69)90033-1

Sudhaus, W., Kiontke, K. and Giblin-Davis, R.M. (2011) Description of *Caenorhabditis angaria* n. sp. (Nematoda: Rhabditidae), an associate of sugarcane and palm weevils (Coleoptera: Curculionidae). *Nematology* 13, 61–78. DOI: 10.1163/138855410X500334

Weakley, B.S. (1981) *A Beginner's Handbook in Biological Transmission Electron Microscopy*, 2nd edn. Churchill Livingstone, Edinburgh, UK.

Webster, P. (2014) Microwave-assisted processing and embedding for transmission electron microscopy. *Methods in Molecular Biology* 1117, 21–37. DOI:10.1007/978-1-62703-776-1_2

Weimer, R.M. (2006) Preservation of *C. elegans* tissue *via* high pressure freezing and freeze-substitution for ultrastructural analysis and immunocytochemistry. *Methods in Molecular Biology* 351, 203–221. DOI: 10.1385/1-59745-151-7:203

Wergin, W.P. (1981) Scanning electron microscopic techniques and applications for use in nematology. In: Zuckerman, B.M. and Rhode, R.A. (eds) *Plant Parasitic Nematodes, Vol. III*. Academic Press, New York, New York, USA, pp. 175–204. DOI: 10.1016/B978-0-12-782203-7.50013-3

Wergin, W.P., Murphy, C.A. and Orion, D. (2000) Using microwave irradiation to improve preservation of female nematodes and gall tissues for TEM observations. *Journal of Nematology* 32, 323–329.

Wharton, D.A. (1991) Freeze-substitution techniques for preparing nematodes for scanning electron microscopy. *Journal of Microscopy* 164, 187–196. DOI: 10.1111/j.1365-2818.1991.tb03207.x

White, J.G., Southgate, E., Thomson, J.N. and Brenner, S. (1986) The structure of the nervous system of the nematode *Caenorhabditis elegans. Philosophical Transactions of the Royal Society B. Biological Sciences* 314, 1–340. DOI: 10.1098/rstb.1986.0056

Wolff, T. (2011) Preparation of *Drosophila* eye specimens for scanning electron microscopy. *Cold Spring Harbor Protocols* 2011, 1383–1385. DOI:10.1101/pdb.prot066506

Wright, K.A. (1991) Nematoda. In: Harrison, F.W. and Ruppert, E.E. (eds) *Microscopic Anatomy of Invertebrates, Vol.* 4. Wiley-Liss, New York, New York, USA, pp. 111–195.

Yeung, E. and Huang, B. (2015) LR White Acrylic Resin. In: Yeung E., Stasolla C., Sumner M., Huang B. (eds) *Plant Microtechniques and Protocols*. Springer, Cham, Switzerland. DOI: 10.1007/978-3-319-19944-3_6

Yushin, V.V., Coomans, A., Borgonie, G. and Malakhov, V.V. (2002) Ultrastructural study of cuticle formation during embryogenesis of the free-living marine nematode *Enoplus demani* (Enoplida). *Invertebrate Reproduction and Development* 42, 189–203. DOI: 10.1080/07924259.2002.9652775

Yushin, V.V., Claeys, M. and Bert, W. (2016) Ultrastructural immunogold localization of major sperm protein (MSP) in spermatogenic cells of the nematode *Acrobeles complexus* (Nematoda, Rhabditida). *Micron* 89, 43–55. DOI: 10.1016/j.micron.2016.07.004

Zhang, G., Sachse, M., Prevost, M.-C., Luallen, R.J., Troemel, E.R. and Félix, M.-A. (2016) A large collection of novel nematode-infecting microsporidia and their diverse interactions with *Caenorhabditis elegans* and other related nematodes. *PLoS Pathogens* 12, e1006093. DOI: 10.1371/journal.ppat.1006093

9 Behavioural and Physiological Assays

ROLAND N. PERRY

University of Hertfordshire, Hatfield, Hertfordshire, UK; Ghent University, Ghent, Belgium

9.1 Introduction

Investigation of the behaviour and physiology of plant-parasitic and free-living nematodes is challenging, mainly because of their microscopic size. Many experimental approaches, particularly the agar plate movement assays, are applicable to vermiform stages of both groups and derive primarily from extensive studies on *Caenorhabditis elegans*. Detailed information on analysing various aspects of the behaviour of *C. elegans* are given in the WormBook (www.wormbook.org) chapter edited by Hart (2006). Behavioural analysis of *Pristionchus pacificus* olfaction is given by Hong (2015). Such techniques are applicable to experiments with vermiform stages of plant-parasitic and free-living nematodes, but others have been developed especially for plant-parasitic nematodes, particularly to understand aspects of the nematode–host relationship. This section will focus on some of the principal techniques for analysing nematode behaviour and physiology.

9.2 Attraction/Repulsion Plate Assays

The most common attraction/repulsion assays are based on Petri dishes containing agar or nematode growth medium where nematodes and test chemicals are added to the medium. The nematodes move along the surface of the medium in response to gradients of non-volatile compounds and the attraction/repulsion can be determined by tracking nematode movement over a set period. Several modifications of the basic technique have been used, including photographing nematode tracks (Riddle and Bird, 1985; Riga and Webster, 1992) and video monitoring of nematode movement (Dusenberry, 1983; Pline and Dusenberry, 1987).

A simple agar plate assay was used by Hewlett *et al.* (1997) to study nematode responses to tannic acid solutions. They used a Petri dish (10 cm diam.) containing 1.7% distilled water agar (15 ml dish). Circular wells (2 cm diam.) were cut out of the agar on opposite sides into which the test compound on one side and control on the other were pipetted and left for 2 days to establish gradients. Nematodes (20 to 30 in 0.05 ml water) were placed at the centre of each plate, which was then kept in the dark at room temperature (24°C).

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
10.1079/9781786391759.0009

After 24 h, the number of nematodes in the attractive and repellent zones was recorded with the use of a counting template. The more complex chemotaxis assay system of Wuyts *et al.* (2006) has been used frequently to monitor movement of plant-parasitic nematodes (Fig. 9.1). Assessment of effects was based on nematode tracks, instead of nematodes themselves, which take into account the movement of the nematodes over the test plates. Plates were also divided in 16 segments instead of four to allow a more precise measurement. The technique of Wuyts *et al.* (2006) is summarized here:

- Fill Petri dishes (5 cm diam.) with 5 ml water agar (0.4%).
- On opposite sides of the plates, make wells of 1 cm diam. Fill wells with 100 µl of the compounds or concentrations to be tested. Leave for gradients to be established (30 min – 3 h) before starting the test.
- Where applicable, use the compound solvent or distilled water (dH_2O) as controls; 1% acetic acid can be used as a standard repellent and 0.5 M $CaCl_2$ as an attractant.
- Pipette test nematodes (usually 10) in the middle of the Petri dish in a minimum volume of water. Keep the plate at constant temperature in the dark for a set period (Wuyts *et al.* (2006) used 1 h for females of *Radopholus similis* and *Pratylenchus penetrans* and 2 h for juveniles of *Meloidogyne incognita*).
- After the set period, stop nematode movement by spraying the plates with ethanol (70%). Movement tracks can then be recorded, for example on film.
- Tracks can be analysed by placing a grid that divides the plates into attractive zones, close to the wells, and repellent zones (Fig. 9.1). For each zone, eight segments are included in the grid. For each segment of the grid, a score is given for the presence (1) or absence of nematode tracks (0). The chemotaxis factor (Cf) can be calculated by dividing the sum of scores of the attractant zones by these of the repellent zones. A Cf greater than 2 means attraction of nematodes, while lower than 0.5 means repellence.
- All such tests should be replicated for each compound/concentration and independently repeated in time.

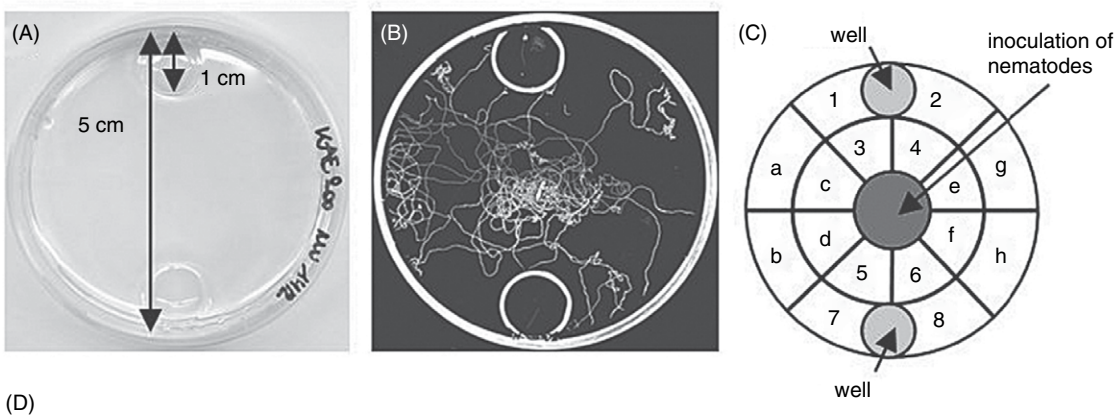

Fig. 9.1. *In vitro* chemotaxis assay. **(A)** Medium on which nematodes come into contact with a concentration gradient of a compound placed in two wells (see C). **(B)** Nematode migration tracks on the medium. **(C)** Grid for the analysis of nematode preferential orientation on the medium; each segment of the grid was given a score for the presence (1) or absence of nematode tracks (0). **(D)** Calculation of the chemotaxis factor (Cf). (From Wuyts *et al*., 2006.)

To examine the response of *M. incognita* second-stage juveniles (J2) to different salts, Qi *et al.* (2015) used 5 cm diam. Petri dishes filled with 0.8% agarose divided into 16 sections in two circles, inner and outer. In the outer circle of each dish, 50 µl of the salt being tested and dH_2O were inoculated on opposite sides and incubated for 1 h at 25°C. Thirty *M. incognita* in 5 µl were placed in the centre of the test arena and incubated at 25°C in the dark for 5 h, after which movement of nematodes was stopped by spraying the plates with ethanol. The numbers of nematodes in the sectors were counted.

Agar plate assays have also been used to examine the effects of binding of antibodies on nematode movement, for example, to the cuticle (Sharon *et al.*, 2002) and amphids (Stewart *et al.*, 1993).

Methods based on movement bioassays have several disadvantages, including the two-dimensional nature of the assay, uncertainty about how quickly a gradient is established, and the concentration to which the nematode is responding is not defined.

9.3 Pluronic Gel Assays

Pluronic F-127 is a non-ionic surfactant, co-polymer of propylene oxide and ethylene oxide containing a trace amount of impurities (Ko and Van Gundy, 1988; Rokhade *et al.*, 2007) and has minimal toxicity, although it has been reported to have adverse effects on some nematodes and plant tissues (Ko and Van Gundy, 1988).

Nematodes can move freely inside the gel in response to gradients, thus making the assay three-dimensional. Pluronic gel is highly transparent, making it easy to monitor nematode movement; it is useful for studying nematode-plant interactions. A 23% solution is a semisolid gel at room temperature but is liquid at temperatures of 15°C and below. Nematodes can be dispersed in the gel at 15°C, a temperature that is not harmful, rather than the higher temperatures required for dispersal in agar. Pluronic gel assays enable attraction of nematodes over time to be quantified and it has been shown to be applicable to a range of plants and nematode species, with studies on host recognition, invasion, development and reproduction, including comparison of infection and development of nematodes on resistant and susceptible plant cultivars (Wang *et al.*, 2009a,b; Dutta *et al.*, 2011; Sasaki-Crawley *et al.*, 2012; Kumari *et al.*, 2016; Pokhare *et al.*, 2019).

Nematodes could be recovered from the gel by filtration after lowering the temperature to 15°C, a property that would be useful for recovering nematodes exposed to roots for making cDNA libraries or other experiments. Pluronic gel is relatively uncontaminated with microbes compared to agar and can be used without autoclaving for short-term experiments but can be autoclaved for assays lasting 3 days or longer (Ko and Van Gundy, 1988).

9.4 Movement Assays

9.4.1 Response to non-volatiles

To test the effects of non-volatile compounds on nematode movement, simple sand tube assays are useful (Evans and Wright, 1982; Clarke and Hennessy, 1987). Polythene tubes (usually, height 1.0 cm) sealed at the base with nylon mesh (53 µm aperture) are filled with sand (particle size 250–600 µm) and placed upright in a small tube or sample jar containing the test solution. The tubes are equilibrated with the test solution by passage of the solution (200 ml) through the tube before use. An aliquot with a known number of nematodes is then pipetted into the top of the tube. The apparatus can be kept at a set temperature and after a set period of time the number of nematodes that migrated through the sand to the reservoir is counted. Acrylic tubes filled with sand were used to establish linear gradients of temperature (Robinson, 1994) and CO_2 (Robinson, 1995) to investigate movement of species of plant-parasitic nematodes. Final distribution of the nematodes was determined by extracting nematodes from sequential slices of sand along the linear gradient.

Sand column assessments of nematode movement preclude direct observation. Microchannel devices have been used with *C. elegans* comprising poly-dimethylsiloxane (PDMS) microchannels on an agar plate, which effectively restrict nematodes to a defined space. These have been used to examine *C. elegans* behaviour (Qin and Wheeler, 2007) and bacterial food preference (Zhang *et al.*, 2005). Otobe *et al.* (2004) used microstructures, consisting of networks of channels of rectangular cross sections (50 µm high, 40–200 µm wide) in

5 mm^2 areas on transparent substrates made of silicon rubber and sealed with a glass plate. This set up was used with different channel dimensions to mimic soil pore spaces and was used to examine the movement and migration of *M. incognita* J2 when the channels were filled with water.

O'Callaghan *et al.* (2018) developed microcosm assays for testing nematode responses. They used transparent soil microcosms, made from microscope slides, a new light sheet imaging technique termed biospeckle selective plane illumination microscopy (BSPIM) for nematode detection, and confocal laser scanning microscopy for high-resolution imaging. BSPIM enabled automatic detection of nematodes in artificial transparent 'soil' (Downie *et al.*, 2012) in the microcosms. Growing plant root systems could be scanned for nematode abundance and activity, and nematode feeding behaviour and responses to various treatments could be observed in soil-like conditions.

9.4.2 Response to volatiles

Volatile signals have been implicated in the ability of parasitic nematodes to locate their hosts. Assays for volatile attractants are more complicated to set up than those for non-volatiles; however, there are several olfactometer assay arenas that are available. The most straightforward are the one-choice point Y olfactometers used by several authors with plant-parasitic nematodes and entomopathogenic nematodes (e.g. Reynolds *et al.* 2011). A Y-tube olfactometer filled with sand was used to examine host-searching behaviour over a 24-h period of infective juveniles of *Heterorhabditis megidis* in the presence of insect hosts and plant roots, either individually or in combination (Boff *et al.*, 2003). Pline and Dusenbery (1987) used a computer tracking system to quantify the responses of *M. incognita* J2 to CO_2, including determining the change in rate of movement.

To test the response of *M. incognita* J2 to plant volatiles, Kihika *et al.* (2017) used a dual choice olfactometer (Fig. 9.2) comprising four components, the stimulus chamber (A) and the control chamber (D) (each 85 mm diam. × 140 mm deep) with a connector (15 mm diam. × 30 mm length) fitted with an ultra-fine mesh screen filled with sterilized sand. The central release arm (B) (20 mm diam. × 60 mm length) was linked to detachable connecting arms (C) (20 mm diam. × 70 mm length) that connected chambers A and D (Fig. 9.2A). For the dual choice olfactometer assays with intact plants, the growth chamber containing 30 plants was paired with a growth chamber containing 300 g moist sand (control). After 4 h (the optimal recovery time)

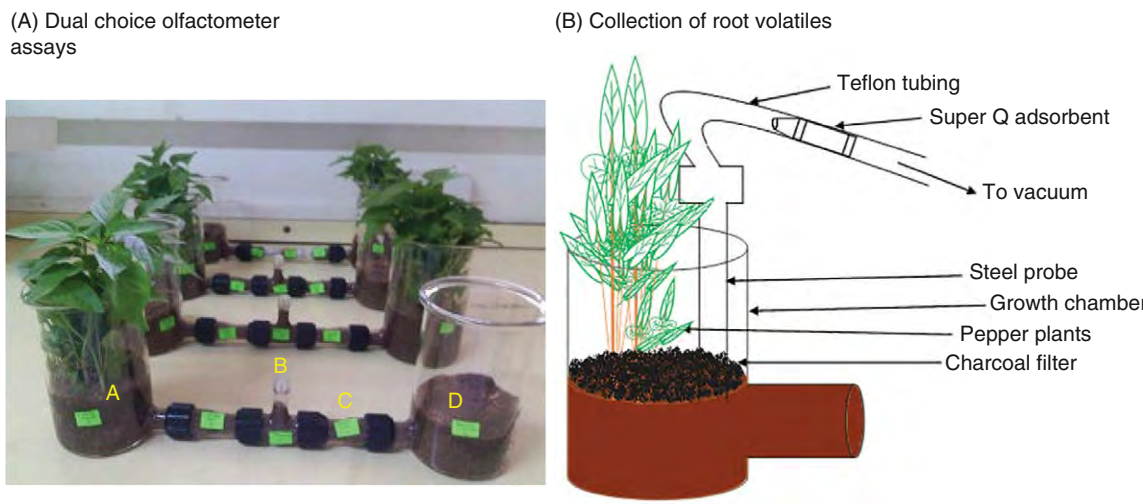

Fig. 9.2. Response of *Meloidogyne incognita* juveniles (J2) to root volatiles of pepper and chemical analysis of the root volatiles. **(A)** Dual choice olfactometer assays to test J2 responses to *Capsicum annum* root volatiles and synthetic blends: A, stimulus chamber; B, release arm; C, connecting arm; and D, control chamber. **(B)** A schematic representation of the volatile collection set-up in the laboratory. (Modified from Kihika *et al.*, 2017.)

the olfactometer was disassembled and the sand in each detachable section was placed on a Baermann funnel to recover and then count the J2. Kihika *et al.* (2017) also used the olfactometer to determine the response of J2 to collected volatiles (Fig. 9.2B). The volatiles were collected for 24 h on a pre-cleaned Super Q (30 mg, Analytical Research System, Gainesville, Florida, USA) adsorbent connected to a steel probe (17 cm long, 0.5 cm internal diam.) inserted in the plant sand root zone in the glass chamber. The probe was connected to a vacuum pump that extracted volatiles from the soil.

9.5 Electrophysiology

Electrophysiology is a useful technique to examine the sensory responses of individual live, intact nematodes but has the disadvantage of requiring expensive equipment. Use of the electrophysiological technique provided detailed analysis of responses to known concentrations of test chemicals (Perry, 2001). A nematode was tethered into a drawn capillary connected to a vacuum pump and the recording electrode was inserted into the cephalic region. The indifferent electrode was placed in the Ringer solution. Electrodes were filled with 0.5 M KC1 and had Ag–AgCl contacts. Resulting electrical signals were amplified, displayed on an oscilloscope and analysed using an AutoSpike program (Syntech). Males (Riga *et al.*, 1996a) and J2 (Rolfe *et al.*, 2000) of *Globodera rostochiensis* and *G. pallida* were exposed to a variety of semiochemicals, including sex pheromones and certain putative phagostimulatory compounds. Use of a perfusion system (Rolfe *et al.*, 2000) enabled sequential exposure of individual nematodes to different test chemicals or to different concentrations of the same chemical. Exposure to 1% DiTera®, a biological nematicide, prevented electrophysiological responses to potato root diffusate (Riga *et al.*, 1996a).

9.6 Stylet Activity

Much information on the electrical events associated with contraction and relaxation of pharyngeal musculature derives from electropharyngeogram (EPG) measurements of the electrical currents that flow in and out of the buccal cavity of *C. elegans* (Raizen and Avery, 1994). Excitation of a pharyngeal muscle causes a pulse of current to flow out of the mouth, resulting in a positive spike in the EPG. Rolfe and Perry (2001) showed that EPG from the anterior end of live, individual J2 of *G. rostochiensis* could be correlated with stylet activity. The indole alkylamine neurotransmitter, serotonin (5-hydroxytryptamine or 5-HT), was used to initiate stylet movement. Concentration-dependent effects can be measured and stylet movement determined accurately over extended periods. Stylet thrusting was significantly reduced by prior exposure of fourth-stage juveniles of *Ditylenchus dipsaci* and J2 of *G. rostochiensis* to 1% DiTera® (Twomey *et al.*, 2002). Tahseen *et al.* (2004) used the EPG technique with adults of six species of free-living nematodes, comparing pharyngeal muscle activity of species with grinders to species without grinders.

9.7 Water Content Changes

There are five basic methods to estimate changes in nematode water content: volumetric, wet and dry weighing, labelled water, interference microscopy and immersion refractometry. Volume measurements have been used as an indirect measure of changes in water content (Wright, 1998) using the equation:

$$V2 / V1 = l2d2^2 / l1d1^2$$

Where V = volume, l = length, and d is the maximum diameter before (1) and after (2) the period in the test solution (after Andrássy, 1956). This method has the advantage of giving continuous readings of volume changes in individual nematodes; however, volume changes in hypertonic solutions may result in uneven collapse of the nematode body, compromising accurate measurements.

Comparison of wet and dry weights requires large masses of nematodes and it can be difficult to determine the wet weight point (Wright, 1998). For example, to determine nematode dry weight Nordmeyer and Dickson (1989) weighed 200,000 J2 in 2 ml dH_2O in an aluminium container. The samples were dried at 120°C for 48 h and weighed immediately after drying. The use of tritiated water (with tritium oxide) by, for

example, Marks *et al.* (1968), also requires large masses of nematodes as the experimental unit. Both weighing and labelled water are discontinuous and destructive methods.

Quantitative experiments to determine the water content of live, individual nematodes can be done using an interference microscope equipped with a fringe field eyepiece. Using monochromatic light, black and white fringes are produced that traverse the nematode under the microscope. The fringes are displaced in the specimen according to the refractive index difference between the specimen and the mounting medium. The refractive index of the nematode is obtained and then the water content can be calculated. Therefore, it is possible to determine the rate at which a nematode gains or loses water. This technique for use with nematodes was first published by Ellenby (1968) and has been used subsequently to determine the water content of nematodes in studies on desiccation survival (Perry, 1999), hatching of cyst nematodes (Perry, 2002), osmotic stress tolerance of intertidal nematodes (Forster, 1998) and moulting (Wright and Perry, 1991). Atkinson and Onwuliri (1981) described a similar technique using an electronic interferometer to determine changes in phase of a laser beam passing through a nematode to give the refractive index of the nematode and, hence, the water content.

Nematode water content can also be determined more simply by a technique similar to immersion refractometry (Ellenby, 1975). If liquid paraffin, for example, is used as the mounting medium the time taken to reach the same refractive index as liquid paraffin (i.e. when the nematode is invisible) can be used as a standard for comparing the rate of drying of different nematodes. A nematode that has the same refractive index as liquid paraffin will have a water content of about 20%.

Distilled water, single salt solutions (e.g. NaCl) and non-electrolytes (e.g. urea and sugars) should be avoided in studies on osmotic and ionic regulation (Wright and Newall, 1980; Wright, 1998). As a hypotonic medium, dH_2O results in water uptake and, thus, is not appropriate for taxonomic studies of nematodes where accurate measurements of dimensions are needed. Information on extracellular ionic concentrations of microscopic nematodes is needed to formulate an accurate Ringer solution but tap water is a reasonable alternative, although the ionic content can vary, even from the same source. Ideally, 'balanced' salt solutions based, for example, on artificial tap water (ATW; see Table 9.1) are recommended. Robinson *et al.* (1984) used a 'synthetic' soil solution containing NaCl and four additional ions (K^+, Ca^{2+}, Mg^{2+}, and NO_3^-) at relative concentrations identical to those in a known soil from Texas, USA. The milligram equivalent concentrations of the six ions were Na^+:K^+:Mg^{2+}:Ca^{2+}:CI^-:NO_3^- = 8:1:6:10:17:8.

Table 9.1. Artificial tap water recipe (from Peter Greenaway, University of New South Wales, Australia, 1975, personal communication).

NaCl: 0.350 mM; KCl: 0.044 mM; Ca $(HCO_3)_2$ mM: 1.0 mM; $Mg(HCO_3)_2$: 0.4 mM (values are mM per litre of distilled water)
As the bicarbonates are not stable, they can be made by bubbling CO_2 through distilled water containing relevant amounts of $CaCO_3$ and MgO; when all solids are dissolved, excess CO_2 is driven off by bubbling air through the solution
The weights needed to make up 1 l are: NaCl: 20.45 mg; KCl: 3.28 mg; $CaCO_3$: 100.09 mg; MgO: 16.12 mg. The calculated osmotic pressure if all salts are ionized is 4.99 mOsm kg^{-1}

9.8 Oxygen Consumption Assays

There is only limited information on the respiration of plant-parasitic and free-living nematodes and much relates to the oxygen consumption rate (OCR), especially to determine the nematicidal effects on respiration; van Aardt *et al.* (2016) give a useful introduction to the literature. It is important to wash samples thoroughly with sterile dH_2O to reduce bacterial contamination immediately before experimentation.

Early work by Nordmeyer and Dickson (1989) used samples of 50,000 J2 concentrated in the 1 ml volume reaction chamber of a Gilson K-1C oxygraph equipped with a Clark electrode. The reaction chamber was maintained at 28°C and the OCR was recorded for 5 min. The nematode samples were stirred at a constant speed with a magnetic stirrer during oxygen measurement to prevent the establishment of oxygen gradients. Stirring at high speeds may damage nematodes but van Aardt *et al.* (2016), using polarographic oxygen sensor and fibre-optic oxygen sensor technology, found no effect of stirring on OCR of *M. incognita* J2. The

fibre-optic oxygen sensor recorded the OCR of as few as five J2. Atkinson (1973) measured the OCR of individual male *Enoplus brevis* and *E. communis* using a constant-temperature microrespirometer, based on a Clark-type oxygen electrode, designed by Atkinson and Smith (1973) for use with small, individual nematodes weighing 60–380 µg.

Dancy *et al.* (2013) detail a protocol for the determination of OCR in living *C. elegans* using the Seahorse Bioscience XF24 Extracellular Flux Analyzer with 24-well plates and between 25 and 150 nematodes per well. After completion of the run, the actual numbers of worms per well were counted from images captured on a camera-fitted dissecting microscope.

9.9 Collecting Female Sex Pheromone, Virgin Females and Males

Secretory–excretory products of female *G. rostochiensis* and *G. pallida*, containing sex pheromones, can be collected using methods described by Riga *et al.* (1996a,b) modified from Greet *et al.* (1968). Potato tubers, 'Desiree', were planted in 10 cm diam. pots in steam-sterilized sand/loam mix. Each pot was inoculated with 30 cysts of *G. rostochiensis* or *G. pallida* and kept in a glasshouse, minimum temperature 18°C. Three-week-old plants were removed from the pots, soil was carefully washed from the roots and the plants were transferred into a plastic bowl containing continuously aerated water. The plants were supported with small canes so that the roots were suspended in the water. Males exit the roots and are unable to locate and fertilize the females as they drop directly to the bottom of the bowl; they were siphoned off and collected in ATW. Males were used for experimentation within 72 h of collection. Females became visible on the roots 4 weeks post-infection. Live white females were collected from the surface of the root using fine forceps and washed in glass distilled water (GDW), and 50 white virgin females were transferred into a watch glass containing 50 ml GDW; this was removed, by pipetting, daily for up to 5 days and stored immediately at 70°C. After concentrating by freeze drying, it was reconstituted for use in 1 ml GDW.

9.10 Viability Tests

There are two main laboratory-based methods for determining the viability of cyst and root-knot nematodes: staining and hatching bioassays.

9.10.1 Staining

9.10.1.1 Vital/non-vital stains

Bird (1979) used fluorescein diacetate on hatched J2 of *M. javanica* and adult females of *C. elegans* as a rapid means of assessing viability, but did not examine unhatched juveniles. Several vital and non-vital stains have been used to determine viability of juveniles within eggs, including chrysoidin (Doliwa, 1956), Nile Blue A (Ogiga and Estey, 1964), phloxine B (Moriarty, 1964) and New Blue R (Southey, 1962). The most consistent and popular stain is Meldola's Blue (Ogiga and Estey, 1964), which has the advantage over phloxine B and Nile Blue A as it is more labile and can be almost completely removed from the eggshell to permit viewing of the unhatched J2. It appears that New Blue R is no longer available and has been superseded by Meldola's Blue.

Kroese *et al.* (2011) examined the viability of *Globodera* spp. using a 96-well assay with a single cyst in each well exposed to 0.05% w/v Meldola's Blue in 100 ml water per well. Assay plates were sealed and incubated at room temperature for 1 week, after which cysts were cut open, eggs were washed out into a 50 ml tube and the percentage stained and non-stained eggs in an aliquot were determined. Eggs that do not stain using Meldola's Blue are considered viable. In the stained eggs of *Globodera*, the inner lipoprotein membranes have broken down, allowing passage of the stain into the dead J2. This method may have an advantage over hatching assays; J2 in diapause will not hatch and the numbers in diapause will not be counted in hatching assays, whereas with staining methods all viable unhatched J2 will not stain. Kroese *et al.* (2011) discuss the suitability of staining and hatching tests for regulatory compared with management requirements.

However, there are reports of too many 'doubtful' eggs, making the Meldola's Blue staining method unreliable. Been and Schomaker (2001) summarized efforts in The Netherlands to overcome this by using a 2-week immersion period and a 0.1% solution followed by a 1-day immersion period in clean water. For nematicide-treated cysts, a 6-month period between treatment and testing was used, as the eggs from young cysts always coloured during this period irrespective of whether they were dead or alive, and not all dead eggs of older cysts stained. Despite these modifications, the method was considered only of use for rapid initial assessments.

Metabolic activity can be determined using the MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), colorimetric assay (reviewed by Berridge *et al.*, 2005). MTT is a tetrazolium dye that undergoes a reduction reaction in metabolically active cells and changes from a weak yellow colour to a dark purple colour as it is converted into an insoluble formazan product. It has been used to determine the viability of *C. elegans* (e.g. James and Davey, 2007). Kearn *et al.* (2017) used the MTT assay to determine mortality and metabolic activity of *G. pallida*. After various test treatments, *G. pallida* J2 were washed in double distilled water (ddH_2O), placed in a 24-well plate in 5 mg ml^{-1} MTT solution made in ddH_2O and gently rotated in the dark for 24 h. Nematodes were then observed under a dissecting microscope and scored for the presence or absence of staining. J2 in control treatment showed dark purple staining at their anterior end, indicating the nematodes were metabolically active and thus viable, whereas nematicidal treatment impaired metabolism and purple staining was not evident.

9.10.1.2 Lipid reserves

The total lipid content of free-living and plant-parasitic nematodes ranges from 11 to 67% of dry weight (Perry *et al.*, 2013). Neutral lipids are the primary energy reserves, and as the amount of lipid and the rate of utilization are related to survival and infectivity, several methods, mostly dye based, have been used to determine neutral lipid reserves of nematodes. Such methods include staining with fat-soluble dyes such as Sudan Black, BODIPY and Oil Red O. Yen *et al.* (2010) found that data from Nile Red and BIODIPY vital labelling do not agree with fixative labelling data using Oil Red O, Sudan Black and Nile Red.

Of these staining methods, Oil Red O has been used most frequently and the standard method was outlined by Storey (1983):

- Dissolve Oil Red O (0.7 g) in absolute propan-2-ol (200 ml), leave overnight, then filter; dilute 180 ml with dH_2O (120 ml).
- Leave the stain overnight at 4°C, then filter it and leave it for 30 min; filter a third time before use.
- Place nematodes in a watch glass, remove excess water and immediately add boiling stain and keep for 30 min at 55°C.
- Transfer nematodes to a watch glass containing equal volumes of glycerin and 70% ethanol; after the alcohol has evaporated, mount nematodes on a slide in 15 µl glycerin.

The stain could be quantified using a scanning microdensitometer to determine optical density (Storey, 1983; Robinson *et al.*, 1987a,b; Fitters *et al.*, 1997). Data obtained are not absolute measurements of the neutral lipid reserves, but the relative values can be used for comparison. Oil Red O was used to measure lipid reserves in J2 of *G. rostochiensis* (Robinson *et al.*, 1985) and *M. exigua* and *M. incognita* (Rocha *et al.*, 2010, 2015) and depletion of reserves was correlated with reduced infectivity. A visual scale rating (6 = full of lipid; 1 = lipid depleted) of Oil Red O has been used as a rapid, field-based assay with J2 of *Meloidogyne* spp. (Christophers *et al.*, 1997).

Shivakumara *et al.* (2019) used fixative-based Nile Red staining of *M. incognita* to determine lipid reserves in relation to infectivity, plus a comparison of different life-cycle stages (Fig. 9.3). Nematodes were concentrated by centrifugation at 106 *g* for 1 min. After aspirating off the supernatant, worms were fixed by adding 100 µl of 40% isopropanol, thoroughly mixed and incubated at room temperate for 5 min. Supernatants were removed and 100 µl of Nile Red solution (stock solution of 5 µl Nile Red in acetone (0.5 mg ml^{-1}) mixed with 1 ml of 40% isopropanol) was added to the nematodes. After gentle rotation at room temperature for 45 min, nematodes were washed and mounted on a glass slide containing a thin layer of 2% water agar (to prevent drying). Fluorescence intensity of micrograph images was quantified.

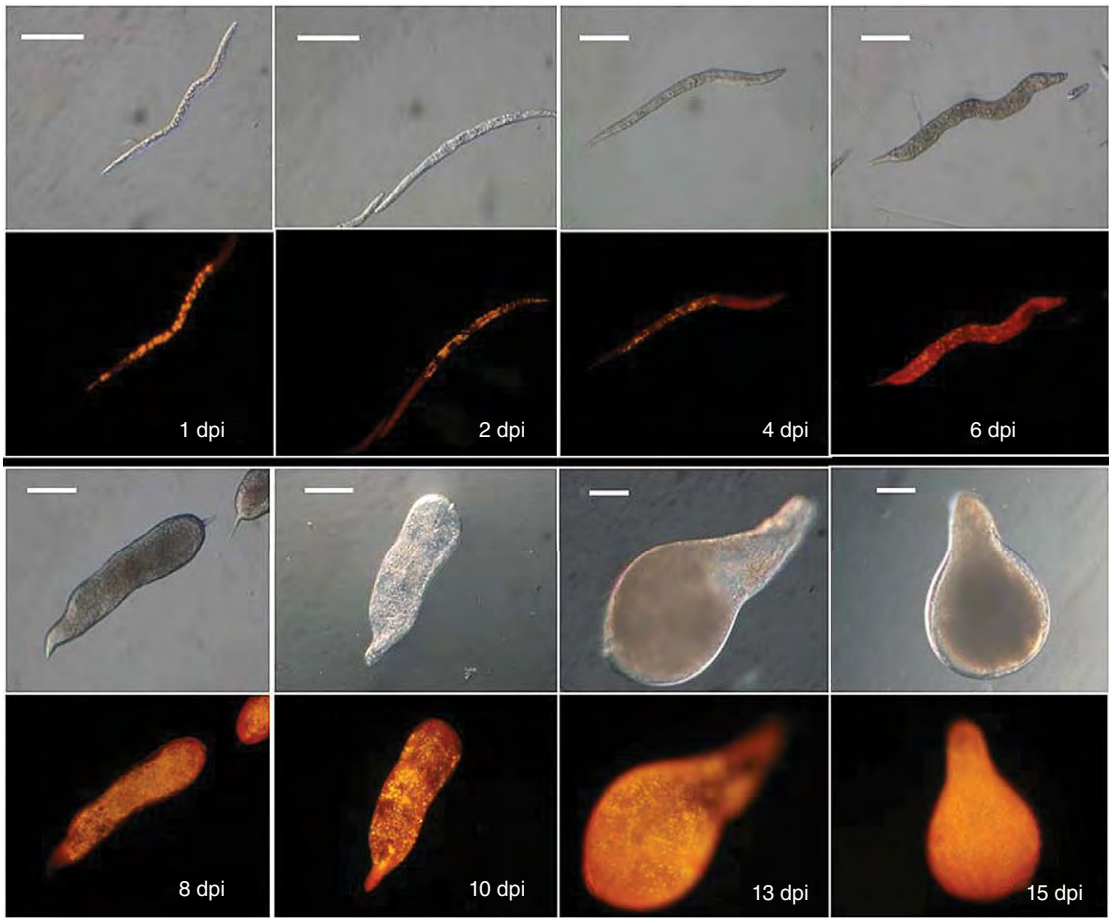

Fig. 9.3. Variation in lipid reserve in different life stages of *Meloidogyne incognita* extracted from infected adzuki bean at 1, 2, 4, 6, 8, 10, 13 and 15 days post inoculation (dpi). Top and bottom panels indicate body dark area and Nile Red stained area of nematodes, respectively. Scale bar = 100 µm. Nile Red that is bound to the fat granules in the hypodermis and intestine/gonad were efficiently and selectively fluoresced in red spectra with excitation/emission wavelength of 553/636 nm in an epifluorescence compound microscope. All images were acquired using identical settings and exposure times for direct comparisons. (From Shivakumara *et al*., 2019.)

Fixative staining methods provide reproducible data but some are prone to errors due to the interference of autofluorescent species and the non-specific staining of cellular structures other than fat stores. Vital dyes with lipid-staining specificity, such as Nile Red, can be used with live worms to assay fat reserves (de Almeida Barros *et al*., 2012) and has been used to assess lipid reserves of *H. schachtii* (Gutbrod *et al*., 2018), *G. pallida* (Kearn *et al*., 2017), *M. incognita* (Marella *et al*., 2013) and *D. dipsaci* (Wharton *et al*., 2008).

A direct, label-free and non-invasive method, termed coherent anti-Stokes Raman scattering (CARS) microscopy, was used by Yen *et al*. (2010) to analyse fat storage in *C. elegans*. CARS imaging provides a direct measure of total fat stores as well as the size, number and lipid-chain unsaturation of individual lipid droplets. Yen *et al*. (2010) found that CARS imaging gives quantification similar to standard biochemical triglyceride quantification and confirmed that feeding worms with vital dyes does not lead to the staining of fat stores, but rather the sequestration of dyes in lysosome-related organelles.

9.10.2 Hatching

Most assays with plant-parasitic nematodes have focused on methods to assess viability and hatching of cyst nematodes. Routine hatching bioassays are time consuming and require large numbers of cysts. Their replacement by a test taking only hours to complete may be desirable; some examples are given below. In addition, hatching assays may not accurately reflect the viability of the population, as some unhatched J2 of species such as *G. pallida* may be in diapause (Perry, 2002), thus being viable but unresponsive to hatching stimuli. Janssen *et al.* (1987) cut cysts from cultures in half and treated them with potato root diffusate (PRD), demonstrating that in cultured cysts, diapause of *G. rostochiensis* and *G. pallida* can be avoided by preventing desiccation. These authors also showed that the artificial hatching procedure to avoid diapause enabled production of five to six generations a year in Petri dishes and three to five generations in pots.

9.10.2.1 Standard hatching assays

Hatching assays can be done using watch glasses to contain the test solution and the biological material. Current hatching assays for cyst nematodes are based on the procedures of Fenwick and Widdowson (1958) and Jones and Gander (1962). Cysts of *Globodera* spp. that have been air-dried should be soaked in water for 7 days before being used in hatching assays. Cysts of other species do not tolerate drying and should be kept moist during extraction.

There are numerous descriptions of hatching units that can be used with cysts or free eggs. Materials such as plastic and glue are not inert and chemicals from these materials could cause contamination. Thus, glass is preferable for constructing a hatching unit. The unit should consist of two compartments, one containing water or the hatching agent, the other containing the test nematodes, separated from each other by a material permeable for water or the hatching agent and the hatched J2.

Variation is reduced the more cysts are used but, obviously, this is conditional on availability of biological material and the time available for counting hatched J2. Usually, 100 cysts per run, split as four batches of 25 cysts is sufficient for biological replication; technical replication is always advisable. The number of J2 that hatch is usually counted once a week for 4 weeks. However, the time selected may vary and the test may continue until hatching has ceased or, for example, <10 J2 hatched; this may take 6–8 weeks or longer. A standard hatching assay using single generation glasshouse-reared cysts of *G. rostochiensis* that have been stored at 5°C for 16 weeks to break the diapause should give 70–80% hatch of juveniles in host root diffusate over a 4-week period (Perry, 2001). Hatch from other species of cyst nematodes in their host root diffusate will vary. If the population of cysts is from the field, including cysts of various ages, then variability of hatch between batches will be greater. If the populations have been treated with nematicides, variability will be even higher and much longer times for the assay will be needed (Been and Schomaker, 2001).

Forrest and Perry (1980) made receptacles for free eggs of *G. rostochiensis* with cylinders of glass tube approximately 2.5 cm long and fixing nylon mesh (aperture 60 µm) at the end with a thin plastic ring. The cylinders were placed in a 20 ml capacity vial. The mesh size was sufficient to retain eggs while allowing passage of hatched J2. Larger tubes can be used for cysts.

Microtitre plates have been used as hatching systems for cysts (for example, Twomey *et al.,* 1995; Byrne *et al.,* 2001; Kroese *et al.*, 2011). Twomey *et al.* (1995) used an incubation unit for *G. rostochiensis* cysts based on a 96-well microtitre plate with wire mesh 'thimbles' in each test well. Each thimble, containing five cysts, fitted into a well, which contained the hatching agent. Hatched J2 moved through the mesh into the well and could be counted. To reduce inter-replicate variability, Twomey *et al.* (1995) graded the cysts through sieves into three size classes: <300 µm diam., 300–500 µm and >500 µm. The juvenile content of cysts in the three classes was determined after the cysts were crushed.

Meloidogyne hatching assays can be undertaken with free eggs or egg masses. Free eggs are obtained by releasing eggs from eggs masses using 0.53% or 1.05% sodium hypochlorite (NaOCl) for 4 min (Hussey and Barker, 1973) or by blending egg masses in 100 ml tap water plus 500 ml 1% NaOCl at high speed for 40 s, followed by sieving and washing (McClure *et al.*, 1973). Egg masses are often difficult to obtain intact. Wesemael *et al.* (2006) used 20 root pieces containing one female and one egg mass of *M. chitwoodi* or

M. fallax for hatching tests. Each mass was put on a 48 µm sieve, covered with 2 ml of the test solution and kept in small plastic bottles fitted with a perforated lid. After hatching had declined to <20 J2 week^{-1}, the remaining eggs were covered with 10% NaOCl and homogenized; the numbers of eggs containing unhatched J2 were counted to determine the percentage hatch.

Counting nematodes under the microscope is laborious and time-consuming and several attempts have been made to develop an accurate automated system. For example, a GOP-302 image analysis system, manufactured by ContextVision, Sweden, was used by Been *et al.* (1996) for automatic counting of the number of hatched J2 of *G. rostochiensis* and *G. pallida* in suspensions. A detailed account of automated methods for counting nematodes is given by Hallmann *et al.*, Chapter 3, this volume.

9.10.2.2 Root diffusates and other hatching agents

Some cyst nematodes, such as *Globodera* spp., are dependent on diffusates from their host plants for substantial hatch; others such as *H. schachtii* hatch well in water (Table 9.2) but root diffusates enhance the rate of hatch. Root diffusate (also termed root exudate or root leachate) for use in hatching bioassays can be obtained from young plants grown in pots (15 cm diam.) containing soil or other growth medium. The common method for obtaining diffusate is based on that detailed by Fenwick (1949) for collection of PRD for hatching potato cyst nematodes.

- Add sufficient water to saturate the pots containing young plants.
- Add a further volume of water (usually 100 ml) and collect the runoff from the pot.
- Filter solution through Whatman No. 1 filter paper.
- For sterile root diffusate pass the solution through a 0.2 µm cellulose acetate filter to retain bacterial and fungal spores (0.2-FW).
- This is the stock solution, which for PRD is usually diluted 1 in 4 with dH_2O to remove the effect of hatch inhibitors. Turner *et al.* (2009) found that half strength PRD consistently stimulated greatest hatch.
- Store the stock solution at about 5°C.

This method provides diffusate for hatching but as the extent of root growth and volume of roots, rather than the hatching activity of PRD *per se*, are important factors in comparing, for example, activity of diffusates from different cultivars, several attempts have been made to quantify activity more accurately. Ellenby (1946) and Forrest and Farrer (1983) placed well-washed roots of pot-grown plants of a known age in a set volume of dH_2O for a set time period to provide the stock solution. PRD is standardized and the possible influence of soil microorganisms is negated. However, comparative data between cultivars may be compromised by variations in root growth. Rawsthorne and Brodie (1986) compared diffusate activity of two potato cultivars and adjusted activity for root weight. Thus, the number of juveniles that hatch in a given period of time per g root weight can be used for comparison. However, root weight may not be as accurate as root

Table 9.2. Grouping of some species of cyst nematodes into four broad categories, based on their hatching response to host root diffusates. (From Perry, 2002.)

Group	Description	Examples
1	Very large numbers of juveniles hatching in response to host root diffusates; few hatching in water	*Globodera rostochiensis*, *G. pallida*, *G. ellingtonae*, *Heterodera cruciferae*, *H. carotae*, *H. goettingiana*, *H. humuli*
2	Very large numbers of juveniles hatching in response to host root diffusates; moderate hatch in water	*H. trifolii*, *H. galeopsidis*, *H. glycines*
3	Very large numbers of juveniles hatching in response to host root diffusates; large hatch in water	*H. schachtii*, *H. avenae*
4	Hatching of juveniles induced by diffusates only in later generations produced during the host growing season; very large hatch in water for all generations	*H. cajani*, *H. sorghi*

Table 9.3. Some artificial hatching agents and the suggested concentrations for common cyst nematodes. (From Shepherd, 1986.)

Species	Compound	Concentration (mM)
Globodera rostochiensis	Sodium metavanadate	0.6
	Picrolonic acid	0.3
G. tabacum	Zinc sulphate	2.0
Heterodera schachtii	Picric acid	3.0
	Zinc chloride	3.0
H. cruciferae	Flavianic acid	0.6
H. glycines	Flavianic acid	3.0
	Zinc chloride	3.0
H. trifolii	Zinc chloride	3.0
H. carotae	Zinc chloride	10.0

length for comparative quantification of diffusate activity. The activity of diffusate also varies with the age of the host plant (Perry, 2002); with cyst nematodes, such as *H. goettingiana*, for example, only hatching in diffusates from plants of a specific age (Perry *et al.*, 1980).

Species of *Meloidogyne* generally hatch well in water without the need for host root diffusate stimulus but Wesemael *et al.* (2006) showed that whilst hatching of *M. chitwoodi* from young plants did not require diffusate stimulus, at the end of the plant growing season egg masses contained a percentage of unhatched J2 that required host root diffusate to cause hatch.

Several artificial hatching agents have been used to induce hatching from *Globodera* and *Heterodera* cysts and freed eggs. The most common ones and the suggested concentrations are given in Table 9.3.

9.10.2.3 Fluorescence assay

Hatching of *G. rostochiensis* involves a change in permeability of the lipoprotein membranes of the eggshell allowing leakage of trehalose out of the egg with concomitant movement of water into the unhatched J2 (Perry, 2002). This can allow the passage of a selected fluorochrome through the eggshell and into the viable unhatched J2, which could be used as a rapid, simple primary screen to evaluate viability and hatching potential of *Globodera* spp. Perry and Feil (1986) used this approach with *G. rostochiensis*. Cysts were cut open to release the eggs, which were rinsed in GDW and added to 6 mm diam. glass cylinders, each with 30 µm mesh nylon netting fixed over the lower end to retain the eggs. Each cylinder was placed in an excavated glass block containing acridine orange dissolved in PRD, made up to a concentration of 0.001%. Eggs were kept in solutions for different time periods (6 min to 24 h), long enough to initiate hatch but too short for actual eclosion to have occurred (Perry and Beane, 1982). Eggs were then rinsed and transferred into a drop of GDW on a glass slide and examined under a fluorescence microscope. Where the acridine orange had passed through the eggshell, the unhatched viable juvenile fluoresced a bright green and could easily be distinguished from non-fluorescing J2 in eggs where there was no uptake of stain. As acridine orange appears not to be capable of penetrating the lipoprotein egg membranes in the absence of a hatching stimulus, such as PRD, Twomey *et al.* (2000) used acridine orange as an indicator of egg permeability in *G. rostochiensis* caused by PRD.

9.10.2.4 Adenosine triphosphate assay

Measurement of adenosine triphosphate (ATP), present only in living tissue, using bioluminescent photometry has been used to determine viability of cyst nematodes (Atkinson and Ballantyne, 1977; Storey and Atkinson, 1979; Storey, 1982). Nematodes are macerated with 0.1 M arsenate buffer at pH4 and 4°C to extract ATP. A photometer is used to measure the ATP content of the cysts by bioluminescence of a luciferin: luciferase extract from fireflies. The bioluminescence the ATP produces is related to the amount of ATP and, therefore, the number of living organisms present. Nematodes must be free of other living material, such as microorganisms and fungi, as ATP from these will interfere with the results.

Huijbregts *et al*. (1996) used HPLC to measure ATP content to estimate viability of field populations of *H. schachtii*. Storey and Marks (1983) used measurement of the ATP content of cysts by bioluminescent photometry as a screen for assessing the resistance of potato clones to *Globodera* spp. The multiplication rates of the nematodes were based on the ratio of ATP levels for the cyst inoculum and the progeny.

9.10.2.5 Trehalose assay

Inside the egg, the J2 of *Globodera* and *Heterodera* spp. is surrounded by perivitelline fluid, which contains trehalose at a concentration of 0.34 M in *G. rostochiensis* (Clarke *et al*., 1978) and 0.5 M in *H. goettingiana* (Perry *et al*., 1980), for example. The presence of trehalose has been used as the basis for a viability assay for *G. rostochiensis* and *G. pallida* (van den Elsen *et al*., 2012) and further evaluation by Ebrahimi *et al*. (2015) has shown this viability assessment to be a robust, rapid technique capable of a minimum detection level of five viable eggs. Van den Elsen *et al*. (2012) considered the limit of detection for regular field samples was ≈10 viable eggs, due to background signals produced by other soil components, and the assay can be combined with a subsequent DNA-based species determination. As trehalose is present in the perivitelline fluid of eggs of cyst nematodes investigated so far (Perry, 2002), it is possible that this method can be used with other cyst nematodes.

9.10.3 Other methods

Luc *et al*. (1969) soaked *Hemicaloosia paradoxa* (= *Hemicycliophora paradoxa*) in a solution of radioactive phosphorus ($^{32}PO_4H_3$) for 24 h and traced the nematode's movement in a layer of soil (0.5 cm thick) using its radioactivity. Mayo and Thomas (1971) labelled *Longidorus elongatus* and *Xiphinema diversicaudatum* by incubation in a dilute aqueous solution of radioactive D-glucose [^{14}C]. In soil, individual nematodes could be detected for at least 12 weeks by autoradiographic techniques.

Using 450–490 nm epi-illumination in a viability assay, Forge and MacGuidwin (1989) found that autofluorescence of live nematodes was restricted to 1.0–5.0 µm diam. globules in the intestinal cells, whereas autofluorescence was dispersed throughout the body in nematodes killed with formaldehyde, freezing or heat.

9.11 References

Andrássy, I. (1956) The determination of volume and weight of nematodes. In: Zuckerman, B.M., Brzeski, M.W. and Deubert, K.H. (eds) *English Translations of Selected East European Papers in Nematology*. University of Massachusetts, East Wareham, Massachusetts, USA, pp. 73–84.

Atkinson, H.J. (1973) The respiratory physiology of the marine nematodes *Enoplus brevis* (Bastian) and *E. communis* (Bastian). *Journal of Experimental Biology* 59, 255–266.

Atkinson, H.J. and Ballantyne, A.J. (1977) The adenosine triphosphate content of cysts of *Globodera pallida* and *G. rostochiensis* as a possible quantitative estimate of field populations. *Annals of Applied Biology* 87, 407–414. DOI: 10.1111/j.1744-7348.1977.tb01905.x

Atkinson, H.J. and Onwuliri, C.O.E. (1981) *Nippostrongylus brasiliensis* and *Haemonchus contortus*: Function of the excretory ampulla of the third-stage larva. *Experimental Parasitology* 52, 191–198.

Atkinson, H.J. and Smith, L. (1973) An oxygen electrode microrespirometer. *Journal of Experimental Biology* 59, 247–253.

Been, T.H. and Schomaker, C.H. (2001) Ways to improve the accuracy of hatching tests for *Globodera* spp. with special emphasis on nematicide trials. *Nematology* 3, 773–795. DOI: 10.1163/156854101753625290

Been, T.H., Meijer, E.M.J., Beniers, A.E. and Knol, J.W. (1996) Using image analysis for counting larvae of potato cyst nematodes (*Globodera* spp.). *Fundamental and Applied Nematology* 19, 297–304.

Berridge, M.V., Herst, P.M. and Tan, A.S. (2005) Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnology Annual Review* 11, 27–152. DOI: 10.1016/S1387-2656(05)11004-7

Bird, A.F. (1979) A method of distinguishing between living and dead nematodes by enzymatically induced fluorescence. *Journal of Nematology* 11, 103–105.

Boff, M.I.C., Zoon, F.C. and Smits, P.H. (2003) Orientation of *Heterorhabditis megidis* to insect hosts and plant roots in a Y-tube sand olfactometer. *Entomologia Experimentalis et Applicata* 98, 329–337. DOI: 10.1046/j.1570-7458.2001.00789.x

Byrne, J.T., Maher, N.J. and Jones, P.W. (2001) Comparative responses of *Globodera rostochiensis* and *G. pallida* to hatching chemicals. *Journal of Nematology* 33, 195–202.
Clarke, A.J. and Hennessy, J. (1987) Hatching agents as stimulants of movement of *Globodera rostochiensis* juveniles. *Revue de Nématologie* 10, 471–476.
Clarke, A.J., Perry, R.N. and Hennessy, J. (1978) Osmotic stress and the hatching of *Globodera rostochiensis*. *Nematologica* 24, 384–392. DOI: 10.1163/187529278X00506
Christophers, A.E.P., Patel, M.N., Benson, J.A., Saka, V.W., Evans, A.A.F. and Wright, D.J. (1997) A rapid field-laboratory bioassay to assess the infectivity of *Meloidogyne* spp. second stage juveniles. *Nematologica* 43, 117–120. DOI: 10.1163/004725997X00089
Dancy, M.D., Kayser, E., Sedensky, M.M. and Morgan, P.G. (2013) Live worm respiration in 24-well format. *The Worm Breeder's Gazette* 19, 11–12.
de Almeida Barros, A.G., Liu, J., Lemieux, G.A., Mullaney, B.C. and Ashrafi, K. (2012) Analyses of *C. elegans* fat metabolic pathways. *Methods in Cell Biology* 107, 383–407. DOI: 10.1016/B978-0-12-394620-1.00013-8
Doliwa, U. (1956) Experimentelle Untersuchungen an Kartoffelnematoden (*Heterodera rostochiensis* Wollenweber). *Wissenschaften Zeitschrift der Universität Rostock* 5, 133–149.
Downie, H., Holden, N., Otten, W., Spiers, A.J., Valentine, T.A. and Dupuy, L.X. (2012) Transparent soil for imaging the rhizosphere. *PLoS One* 7, e44276. DOI: 10.1371/journal.pone.0044276
Dusenbery, D.B. (1983) Chemotactic behavior of nematodes. *Journal of Nematology* 15, 168–173.
Dutta, T.K., Powers, S.J., Kerry, B.R., Gaur, H.S. and Curtis, R.H.C. (2011) Comparison of host recognition, invasion, development and reproduction of *Meloidogyne graminicola* and *M. incognita* on rice and tomato. *Nematology* 13, 509–520.
Ebrahimi, N., Viaene, N. and Moens, M. (2015) Optimizing trehalose-based quantification of live eggs in potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*). *Plant Disease* 99, 947–953. DOI: 10.1094/PDIS-09-14-0940-RE
Ellenby, C. (1946) The influence of potato variety on the cyst of the potato root eelworm, *Heterodera rostochiensis* Wollenweber. *Annals of Applied Biology* 33, 433–446. DOI: 10.1111/j.1744-7348.1946.tb06335.x
Ellenby, C. (1968) Determination of the water content of nematode worms by interference microscopy. *Experientia* 24, 84–85. DOI: 10.1007/BF02136811
Ellenby, C. (1975) A simple method of comparing the rate of drying of nematode worms. *Nematologica* 21, 115–116. DOI: 10.1163/187529275X00455
Evans, S.G. and Wright, D.J. (1982) Effects of the nematicide oxamyl on life cycle stages of *Globodera rostochiensis*. *Annals of Applied Biology* 100, 511–519. DOI: 10.1111/j.1744-7348.1982.tb01417.x
Fenwick, D.W. (1949) Investigations on the emergence of larvae from cysts of the potato-root eelworm *Heterodera rostochiensis*. I. Technique and variability. *Journal of Helminthology* 23, 151–170. DOI: 10.1017/S0022149X0003248X
Fenwick, D.W. and Widdowson, E. (1958) The conduct of hatching tests on cysts of the potato-root eelworm *Heterodera rostochiensis* (Woll.). *Journal of Helminthology* 32, 125–134. DOI: 10.1017/S0022149X00019532
Fitters, P.F.L., Meijer, E.M.J., Wright, D.J. and Griffin, C.T. (1997) Estimation of lipid reserves in unstained living and dead nematodes by image analysis. *Journal of Nematology* 29, 160–167.
Forge, T.A. and MacGuidwin, A.E. (1989) Nematode autofluorescence and its use as an indicator of viability. *Journal of Nematology* 21, 399–403.
Forrest, J.M.S. and Farrer, L.A. (1983) The response of eggs of the white potato cyst nematode *Globodera pallida* to diffusate from potato and mustard roots. *Annals of Applied Biology* 103, 283–289. DOI: 10.1111/j.1744-7348.1983.tb02766.x
Forrest, J.M.S. and Perry, R.N. (1980) Hatching of *Globodera pallida* eggs after brief exposures to potato root diffusate. *Nematologica* 26, 130–132. DOI: 10.1163/187529280X00639
Forster, S.J. (1998) Osmotic stress tolerance and osmoregulation of intertidal and subtidal nematodes. *Journal of Experimental Marine Biology and Ecology* 224, 109–125. DOI: 10.1016/S0022-0981(97)00192-5
Greet, D.N., Green, C.D. and Poulton, M.E. (1968) Extraction, standardisation and assessment of the volatility of the sex attractants of *Heterodera rostochiensis* and *H. schachtii*. *Annals of Applied Biology* 65, 511–519. DOI: 10.1111/j.1744-7348.1968.tb04553.x
Gutbrod, P., Gutbrod, K., Nauen, R., Elashry, A., Siddique, S., Benting, J., Dormann, P. and Grundler, F.M.W. (2018) Inhibition of acetyl-CoA carboxylase by spirotetramat causes lipid depletion and surface coat deficiency in nematodes. *bioRxiv* preprint (March 7, 2018). DOI: 10.1101/278093
Hart, A.C. (ed.) (2006) Behavior. In: *WormBook*. DOI: 10.1895/wormbook.1.87.1. Available at: http://www.wormbook.org (accessed 11 February 2020).
Hewlett, T.E., Hewlett, E.M. and Dickson, D.W. (1997) Response of *Meloidogyne* spp., *Heterodera glycines*, and *Radopholus similis* to tannic acid. *Journal of Nematology* 29, 737–741.

Hong, R.A. (2015) *Pristionchus pacificus* olfaction. In: Sommer, R.J. (ed.) Pristionchus pacificus. *A nematode model for comparative and evolutionary biology. Nematology Monographs and Perspectives 11* (Series editors: Hunt, D.J. and Perry, R.N.). Brill, Leiden, The Netherlands, pp. 331–352. DOI: 10.1163/9789004260306_013

Huijbregts, A.W.M., Gijssel, P.D., Munning, R.G. and Heijbroek, W. (1996) Estimation of the field population and the vitality of *Heterodera schachtii*, measuring ATP, ADP and AMP contents of eggs and juveniles by HPLC. *Communications in Soil Science and Plant Analysis* 27, 1153–1168. DOI: 10.1080/00103629609369623

Hussey, R.S. and Barker, K.R. (1973) A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Disease Reporter* 57, 1025–1028.

James, C.E. and Davey, M.W. (2007) A rapid colorimetric assay for the quantitation of the viability of free-living larvae of nematodes *in vitro*. *Parasitology Research* 101, 975–980. DOI: 10.1007/s00436-007-0572-1

Janssen, R., Bakker, J. and Gommers, F.J. (1987) Circumventing the diapauses of potato cyst nematodes. *Netherlands Journal of Plant Pathology* 83, 107–113. DOI: 10.1007/BF02000561

Jones, F.G.W. and Gander, M.C. (1962) A bioassay for *Heterodera* spp. without counting cysts or larvae. *Nematologica* 8, 39–50. DOI: 10.1163/187529262X01007

Kearn, J., Lilley, C., Urwin, P., O'Connor, V. and Holden-Dye, L. (2017) Progressive metabolic impairment underlies the novel nematicidal action of fluensulfone on the potato cyst nematode *Globodera pallida*. *Pesticide Biochemistry and Physiology* 142, 83–90. DOI: 10.1016/j.pestbp.2017.01.009

Kihika, R., Murungi, L.K., Coyne, D., Ng'ang'a, M., Hassanali, A., Teal, P.E.A. and Torto, B. (2017) Parasitic nematode *Meloidogyne incognita* interactions with different *Capsicum annum* cultivars reveal the chemical constituents modulating root herbivory. *Scientific Reports* 7, 2903. DOI: 10.1038/s41598-017-02379-8

Ko, M.P. and Van Gundy, S.D. (1988) An alternative gelling agent for culture and studies of nematodes, bacteria, fungi, and plant tissues. *Journal of Nematology* 20, 478–485.

Kroese, D., Zasada, I.A. and Ingham, R.E. (2011) Comparison of Meldola's Blue staining and hatching assay with potato root diffusate for assessment of *Globodera* sp. egg viability. *Journal of Nematology* 43, 182–186.

Kumari, C., Dutta, T.K., Banakar, P. and Rao, U. (2016) Comparing the defence-related gene expression changes upon root-knot nematode attack in susceptible *versus* resistant cultivars of rice. *Scientific Reports* 6, 22846. DOI: 10.1038/srep22846

Luc, M., Lespinat, P. and Souchaud, B. (1969) Marquage direct de *Hemicycliophora paradoxa* par le phosphore radioactif. Utilisation pour l'étude des désplacements des nématodes phytoparasites dans le sol. *Nematologica* 15, 35–43. DOI: 10.1163/187529269X00074

Marella, H.H., Nielsen, E., Schachtman, D.P. and Taylor, C.G. (2013) The amino acid permeases AAP3 and AAP6 are involved in root-knot nematode parasitism of *Arabidopsis*. *Molecular Plant-Microbe Interactions*, 26, 44–54. DOI: 10.1094/MPMI-05-12-0123-FI

Marks, C.F., Thomason, I.J. and Castro, C.F. (1968) Dynamics of the permeation of nematodes by water, nematocides and other substances. *Experimental Parasitology* 22, 321–337. DOI: 10.1016/0014-4894(68)90109-4

Mayo, M.A. and Thomas, P.R. (1971) Labelling of *Longidorus elongatus* and *Xiphinema diversicaudatum* with glucose [^{14}C]. *Nematologica* 17, 23–28. DOI: 10.1163/187529271X00378

McClure, M.A., Kruk, T.H. and Misaghi, I. (1973) A method for obtaining quantities of clean *Meloidogyne* eggs. *Journal of Nematology* 5, 230.

Moriarty, F. (1964) The efficacy of Chrysoidin, New Blue R and Phloxine B for determining the viability of beet eelworm, *Heterodera schachtii* Schm. *Nematologica* 10, 644–646. DOI: 10.1163/187529264X00295

Nordmeyer, D. and Dickson, D.W. (1989) Effect of carbamate, organophosphate, and avermectin nematicides on oxygen consumption by three *Meloidogyne* spp. *Journal of Nematology* 21, 472–476.

O'Callaghan, F.E., Braga, R.A., Neilson, R., MacFarlane, S.A. and Dupuy, L.X. (2017) New live screening of plant-nematode interactions in the rhizosphere. *Scientific Reports* 8, 1440. DOI: 10.1038/s41598-017-18797-7

Ogiga, I.R. and Estey, R.H. (1974) The use of Meldola Blue and Nile Blue A, for distinguishing dead from living nematodes. *Nematologica* 20, 271–276. DOI: 10.1163/187529274X00302

Otobe, K., Itou, K. and Mizukubo, T. (2004) Micro-moulded substrates for the analysis of structure-dependent behaviour of nematodes. *Nematology* 6, 73–77. DOI: 10.1163/156854104323072946

Perry, R.N. (1999) Desiccation survival of parasitic nematodes. *Parasitology* 119, S19–S30. DOI: 10.1017/S0031182000084626

Perry, R.N. (2001) Analysis of the sensory responses of parasitic nematodes using electrophysiology. *International Journal for Parasitology* 31, 909–918. DOI: 10.1016/S0020-7519(01)00198-9A

Perry, R.N. (2002) Hatching. In: Lee, D.L. (ed.) *The Biology of Nematodes*. Taylor and Francis, London, UK.

Perry, R. N. and Bean, J. (1982) The effect of brief exposures to potato root diffusate on the hatching of *Globodera rostochiensis*. *Revue de Nématologie* 5, 221–224.

Perry, R.N. and Feil, J.J. (1986) Observations on a novel hatching bioassay for *Globodera rostochiensis* using fluorescence microscopy. *Revue de Nématologie* 9, 280–282.
Perry, R.N., Clarke, A.J. and Beane, J. (1980) Hatching of *Heterodera goettingiana in vitro*. *Nematologica* 26, 493–495. DOI: 10.1163/187529280X00422
Perry, R.N., Wright, D.J. and Chitwood, D.J. (2013) Reproduction, physiology and biochemistry. In: Perry, R.N. and Moens, M. (eds). *Plant Nematology*, 2nd edn. CAB International, Wallingford, UK, pp. 219–245.
Pline, M. and Dusenbery, D.B. (1987) Responses of the plant-parasite nematode *Meloidogyne incognita* to carbon dioxide determined by video camera-computer tracking. *Journal of Chemical Ecology* 13, 873–888. DOI: 10.1007/BF01020167
Pokhare, S.S., Habash, S.S., Jones, J., Elashry, A. and Grundler, F.W.M. (2019) *In vitro* life cycle of *Heterodera sacchari* on pluronic gel. *Nematology* 21, 573–579. DOI: 10.1163/15685411-00003236
Qi, Y.H., Meng, L., Cao, S.F., Li, M.Q., Chen, S.L. and Ye, D.Y. (2015) Chemotaxis of *Meloidogyne incognita* in response to different salts. *Agricultural Sciences* 6, 900–907. DOI: 10.4236/as.2015.69086
Qin, J. and Wheeler, A.R. (2007) Maze exploration and learning in *C. elegans*. *Lab on a Chip* 2, 186–192. DOI: DOI: 10.1039/b613414a
Raizen, D.M. and Avery, L. (1994) Electrical activity and behaviour in the pharynx of *Caenorhabditis elegans*. *Neuron* 12, 483–495.
Rawsthorne, D. and Brodie, B.B. (1986) Relationships between root growth of potato, root diffusate production and hatching of *Globodera rostochiensis*. *Journal of Nematology* 18, 379–384.
Reynolds, A.M., Dutta, T.K., Curtis, R.H.C., Powers, S.J., Gaur, H.S. and Kerry, B.R. (2011) Chemotaxis can take plant-parasitic nematodes to the source of a chemo-attractant via the shortest possible routes. *Journal of the Royal Society Interface* 8, 568–577. DOI: 10.1098/rsif.2010.0417
Riddle, D.L. and Bird, A.F. (1985) Responses of the plant parasitic nematodes *Rotylenchulus reniformis*, *Anguina agrostis* and *Meloidogyne javanica* to chemical attractants. *Journal of Nematology* 91, 185–195.
Riga, E. and Webster, J.M. (1992) Use of sex pheromones in the taxonomic separation of *Bursaphelenchus* spp. (Nematoda), pathogens of pine trees. *Nematologica* 38, 133–145. DOI: 10.1163/187529292X00126
Riga, E., Perry, R.N., Barrett, J. and Johnston, M.R.L. (1996a) Electrophysiological responses of males of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida* to their sex pheromones. *Parasitology* 112, 239–246. DOI: 10.1017/S0031182000084821
Riga, E., Perry, R.N. and Barrett, J. (1996b). Electrophysiological analysis of the response of males of *Globodera rostochiensis* and *G. pallida* to their female sex pheromones and to potato root diffusate. *Nematologica* 42, 493–498. DOI: 10.1163/004525996X00091
Robinson, A.F. (1994) Movement of five nematode species through sand subjected to natural temperature gradient fluctuations. *Journal of Nematology* 26, 46–58.
Robinson, A.F. (1995) Optimal release rates for attracting *Meloidogyne incognita*, *Rotylenchulus reniformis*, and other nematodes to carbon dioxide in sand. *Journal of Nematology* 27, 42–50.
Robinson, A.F., Orr, C.C. and Heintz, C.E. (1984) Activity and survival of *Orrina phyllobia*: preliminary investigations on the effects of solutes. *Journal of Nematology* 16, 26–30.
Robinson, M.P., Atkinson, H.J. and Perry, R.N. (1985) The effect of delayed emergence on the subsequent infectivity of second stage juveniles of the potato cyst nematode *Globodera rostochiensis*. *Nematologica* 31, 171–178.
Robinson, M.P., Atkinson, H.J. and Perry, R.N. (1987a) The influence of temperature on the hatching, activity and lipid utilization of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. *Revue de Nématologie* 10, 349–354.
Robinson, M.P., Atkinson, H.J. and Perry, R.N. (1987b) The influence of soil moisture and storage time on the motility, infectivity and lipid utilization of second stage juveniles of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. *Revue de Nématologie* 10, 343–348.
Rocha, F.S., Campos, V.P. and de Souza, J.T. (2010) Variation in lipid reserves of second-stage juveniles of *Meloidogyne exigua* in a coffee field and its relationship with infectivity. *Nematology* 12, 365–371. DOI: 10.1163/138855409X12548945788367
Rocha, F.S., Campos, V.P., Catão, H.C.R.M., Muniz, M.D.F.S. and Civil, N. (2015) Correlations among methods to estimate lipid reserves of second-stage juveniles and its relationships with infectivity and reproduction of *Meloidogyne incognita*. *Nematology* 17, 345–352. DOI: 10.1163/15685411-00002871
Rokhade, A., Shelke, N., Patil, S. and Aminabhavi, T. (2007) Novel hydrogel microspheres of chitosan and pluronic F-127 for controlled release of 5-fluorouracil. *Journal of Microencapsulation* 24, 274–288.
Rolfe, R.N. and Perry, R.N. (2001) Electropharyngeograms and stylet activity of second stage juveniles of *Globodera rostochiensis*. *Nematology* 3, 31–34. DOI: 10.1163/156854101300106865
Rolfe, R.N., Barrett, J. and Perry, R.N. (2000) Analysis of chemosensory responses of second stage juveniles of *Globodera rostochiensis* using electrophysiological techniques. *Nematology* 2, 523–533. DOI: 10.1163/156854100509448

Sasaki-Crawley, A., Curtis, R., Birkett, M., Papadopoulos, A., Blackshaw, R. and Pickett, J. (2012) The use of Pluronic F-127 to study the development of the potato cyst nematode, *Globodera pallida*. *Nematology* 14, 869–873. DOI: 10.1163/156854112X631944

Sharon, E., Spiegel, Y., Salomon, R. and Curtis, R.H.C. (2002) Characterization of *Meloidogyne javanica* surface coat with antibodies and their effect on nematode behaviour. *Parasitology* 125, 177–185. DOI: 10.1017/S0031182002001907

Shepherd, A.M. (1986) Extraction and estimation of cyst nematodes. In: Southey, J.F. (ed.) *Laboratory Methods for Work with Plant and Soil Nematodes* 6th edn. Her Majesty's Stationery Office, London, UK, pp. 31–49.

Shivakumara, T.N., Dutta, T.K., Mandal, A. and Rao, U. (2019) Estimation of lipid reserves in different life stages of *Meloidogyne incognita* using image analysis of Nile Red-stained nematodes. *Nematology* 21, 267–274. DOI: 10.1163/15685411-00003212

Southey, J.F. (1962) New blue R, a stain that differentiates between living and dead nematodes. *Nematologica* 8, 201–208. DOI: 10.1163/187529262X00431

Stewart, G.R., Perry, R.N. and Wright, D.J. (1993) Studies on the amphid specific glycoprotein gp32 in different life cycle stages of *Meloidogyne* species. *Parasitology* 107, 573–578. DOI: 10.1017/S0031182000068165

Storey, R.M.J. (1982) The ATP method for rapid assessment of the efficacy of a single application of a fumigant against *Globodera* spp. in field soils. *Annals of Applied Biology* 101, 93–98. DOI: 10.1111/j.1744-7348.1982.tb00804.x

Storey, R.M.J. (1983) The initial neutral lipid reserves of juveniles of *Globodera* spp. *Nematologica* 29, 144–150. DOI: 10.1163/187529283X00410

Storey, R.M.J. and Atkinson, H.J. (1979) Measurement of the adenosine triphosphate content of cysts of *Globodera rostochiensis* as a method for assessing the efficacy of fumigant nematicides. *Annals of Applied Biology* 93, 299–304. DOI: 10.1111/j.1744-7348.1979.tb06545.x

Storey, R.M.J. and Marks, R.J. (1983) Screening for resistance to potato cyst nematodes using bioluminescent photometry. *Annals of Applied Biology* 103, 321–326. DOI: 10.1111/j.1744-7348.1983.tb02770.x

Tahseen, Q., Sheridan, J. and Perry, R.N. (2004) Electropharyngeograms of pharyngeal pumping activity in six species of free-living nematodes. *Nematology* 6, 49–54. DOI: 10.1163/156854104323072919

Turner, S.J., Fleming, C.C., Moreland, B.P. and Martin, T.J.G. (2009) Variation in hatch among pathotypes of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, in response to potato root diffusate from *Solanum* spp. I. Preliminary assessments to establish optimal testing conditions. *Nematology* 11, 749–756. DOI: 10.1163/156854109X411005

Twomey, U., Raftery, T., Devine, K. and Jones, P. (1995) An improved procedure for assaying hatching activity of potato root diffusates towards *Globodera rostochiensis*. *Nematologica* 41, 258–268. DOI: 10.1163/003925995X00224

Twomey, U., Warrior, P., Kerry, B.R. and Perry, R.N. (2000) Effects of the biological nematicide, DiTera®, on hatching of *Globodera rostochiensis* and *G. pallida*. *Nematology* 2, 355–362. DOI: 10.1163/156854100509114

Twomey, U., Rolfe, R.N., Warrior, P. and Perry, R.N. (2002) Effects of the biological nematicide, DiTera®, on movement and sensory responses of second stage juveniles of *Globodera rostochiensis*, and stylet activity of *G. rostochiensis* and fourth stage juveniles of *Ditylenchus dipsaci. Nematology* 4, 909–915. DOI: 10.1163/156854102321122520

van Aardt, W.J., Fourie, H., Pretorius, M., Louw, R. and van Dyk, H. (2016) The effects of stirring, population levels and a potential anti-nematodal product on the respiration of second-stage juveniles of *Meloidogyne incognita* measured with different technology. *Nematology* 18, 1053–1061. DOI: 10.1163/15685411-00003014

van den Elsen, S., Ave, M., Schoenmakers, N., Landeweert, R., Bakker, J. and Helder, J. (2012) A rapid, sensitive, and cost-efficient assay to estimate viability of potato cyst nematodes. *Phytopathology* 102, 140–146. DOI: 10.1094/PHYTO-02-11-0051

Wang, C., Lower, S. and Williamson, V.M. (2009a) Application of Pluronic gel to the study of root-knot nematode behaviour. *Nematology* 11, 453–464. DOI: 10.1163/156854109X447024

Wang, C., Bruening, G. and Williamson, V.M. (2009b) Determination of preferred pH for root-knot nematode aggregation using pluronic F-127 gel. *Journal of Chemical Ecology* 35, 1242–1251. DOI: 10.1007/s10886-009-9703-8

Wesemael, W.M.L., Perry, R.N. and Moens, M. (2006) The influence of root diffusate and host age on hatching of the root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax. Nematology* 6, 895–902. DOI: 10.1163/156854106779799204

Wharton, D.A., Petrone, L., Duncan, A. and McQuillan, A.J. (2008) A surface lipid may control the permeability slump associated with entry into anhydrobiosis in the plant parasitic nematode *Ditylenchus dipsaci*. *Journal of Experimental Biology* 211, 2901–2908. DOI: 10.1242/jeb.020743

Wright, D.J. (1998) Respiratory physiology, nitrogen excretion and osmotic and ionic regulation. In: Perry, R.N. and Wright, D.J. (eds) *The Physiology and Biochemistry of Free-living and Plant-parasitic Nematodes*. CABI Publishing, Wallingford, UK, pp.103–131.

Wright, D.J. and Newall, D.R. (1980) Osmotic and ionic regulation in nematodes. In: Zuckerman, B.M. (ed.) *Nematodes as Biological Models*, Vol. 2, Academic Press, New York New York, USA, pp. 143–164.

Wright, K. and Perry, R.N. (1991) Moulting of *Aphelenchoides hamatus*, with especial reference to the formation of the stomatostyle. *Revue de Nématologie* 14, 497–504.
Wuyts, N., Swennen, R. and De Waele, D. (2006) Effects of plant phenylpropanoid pathway products and selected terpenoids and alkaloids on the behaviour of the plant-parasitic nematodes *Radopholus similis*, *Pratylenchus penetrans* and *Meloidogyne incognita*. *Nematology* 8, 89–101. DOI: 10.1163/156854106776179953
Yen, K., Le, T.T., Bansal, A., Narasimhan, S.D., Cheng, J.X. and Tissenbaum, H.A. (2010) A comparative study of fat storage quantitation in nematode *Caenorhabditis elegans* using label and label-free methods. *PloS One* 5, e12810.
Zhang, Y., Lu, H. and Bargmann, C.I. (2005) Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. *Nature* 438, 179–184. DOI: 10.1038/nature04216

10 Staining Chromosomes

JONATHAN D. EISENBACK

Virginia Tech, Blacksburg, Virginia, USA

10.1 Introduction

Chromosomes of nematodes are stained in order to count their number and determine the mode of reproduction from their behaviour during meiosis (Triantaphyllou, 1985a). Chromosomes of plant-parasitic nematodes are extremely small and are often difficult to count so that a good stain is very important for proper imaging of these structures. Nuclear stains that have been used to observe chromosomes of nematodes include the fluorescent DAPI (4'6-diamidino-2-phenylindole) (Hasegawa *et al*., 2006; Yoshida *et al*., 2009) and Feulgen, Giemsa and the fluorescent stain, Hoechst 33258 (Bisbenzimide) (Grisi *et al*., 1995). Although several different stains have been utilized for staining chromosomes of nematodes, propionic-orcein stain has been judged by Grisi *et al*. (1995), working with *Globodera pallida*, to be the best.

Root-knot nematodes induce repeated mitosis without cytokinesis in their host to form specialized feeding sites (Vieira and Engler, 2015). Other nematodes also affect the number of nuclei in the tissues that have become feeding sites (Sobczak and Golinowski, 2009). Observation of these specialized feeding sites may increase our understanding of the host parasite relationship, which may eventually lead to control tactics.

In this chapter, the basic information for staining chromosomes of *Meloidogyne* and free-living nematodes is presented, together with information on staining giant cells and syncytia.

10.2 Staining Chromosomes of Root-knot Females

The number and behaviour of the chromosomes of the root-knot nematodes, *Meloidogyne* spp., are useful to identify species, of which several occur as cytological races (Triantaphyllou, 1981).

10.2.1 Propionic-orcein staining of root-knot females

10.2.1.1 Preparation of propionic-orcein stain

Triantaphyllou (1985a) optimized the propionic-orcein staining method for the root-knot nematodes (Fig. 10.1). This method combines 2.2 g orcein stain (natural or synthetic) with 100 ml glacial propionic acid that is boiled gently for 20–60 min (**caution:** boiling can suddenly become violent; use small glass beads). One of several types of glass condensers is used to recover the vapours that are being boiled away (Fig. 10.2). The stain is cooled to room temperature and diluted with 100 ml of distilled water. It is filtered through a fine filter paper and stored in a dark brown glass bottle that is capped with a ground glass stopper and pipette.

 Techniques for Work with Plant and Soil Nematodes
(eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
10.1079/9781786391759.0010

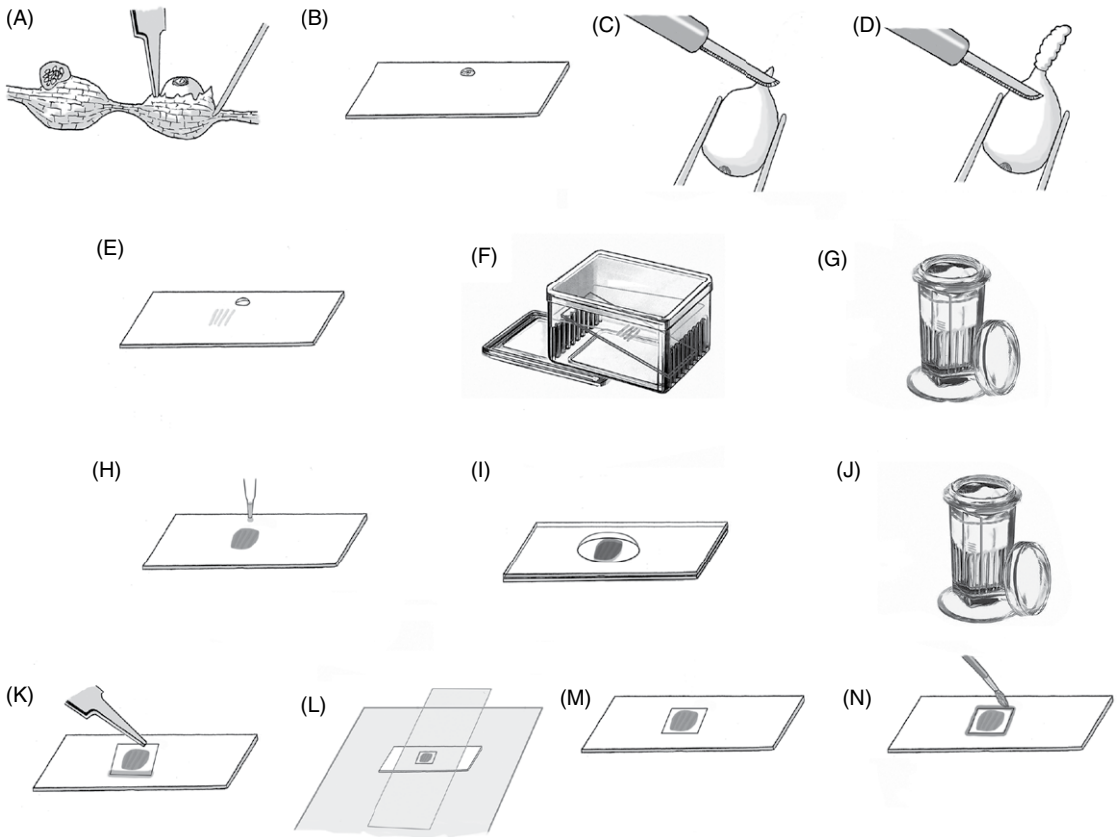

Fig. 10.1. Major steps in the technique for staining chromosomes of root-knot nematodes with propionic-orcein acid stain. **(A)** Remove mature, white females from a 45-day-old culture with a dissecting needle and forceps. **(B)** Pipette four or five females with a small drop of water onto the edge of a glass slide. **(C)** Cut off the head of the female with a scalpel or eye-knife. **(D)** Squeeze the body contents out in a straight line on the slide perpendicular to the length of the slide. **(E)** Add three or four specimens with their contents separated from each other. **(F)** Place the slide with tissue side down into a rectangular staining dish for hydrolysis. **(G)** Place the slide, with tissue facing toward you, into a staining jar for fixation. **(H)** Place a small drop of propionic-orcein stain onto the tissue. **(I)** Cover the stain and material with an inverted cavity slide. **(J)** Destain the material in a staining jar. **(K)** Cover the material with a cover slip containing a small drop of acetic acid. **(L)** Blot the preparation dry with a tissue paper, being careful not to disturb the cover slip. **(M)** Air dry the slide. **(N)** Seal the cover slip with nail polish or a wax mixture.

10.2.1.2 Selecting and obtaining nematode material for cytological study

Live, young, egg-producing females developing in the roots of very favourable host plants are selected from glasshouse cultures grown at 23–28°C that have been properly watered, fertilized and maintained pest-free and are the best source of nematode material; usually young females with creamy yellowish to white egg masses containing 100–300 eggs are ideal (45-day old cultures).

Wash the roots of infected glasshouse plants shortly before extraction of females (see Viaene *et al.*, Chapter 2, this volume). If extraction of females has to be delayed for a few hours or overnight, keep the roots wet in plastic bags at room temperature; do not refrigerate them. Extract females from the galled roots using forceps and a dissecting needle, or a surgical eye-knife under a stereoscope at 10–15× magnification. Females can be extracted directly from the wet roots and transferred immediately into a BPI dish containing

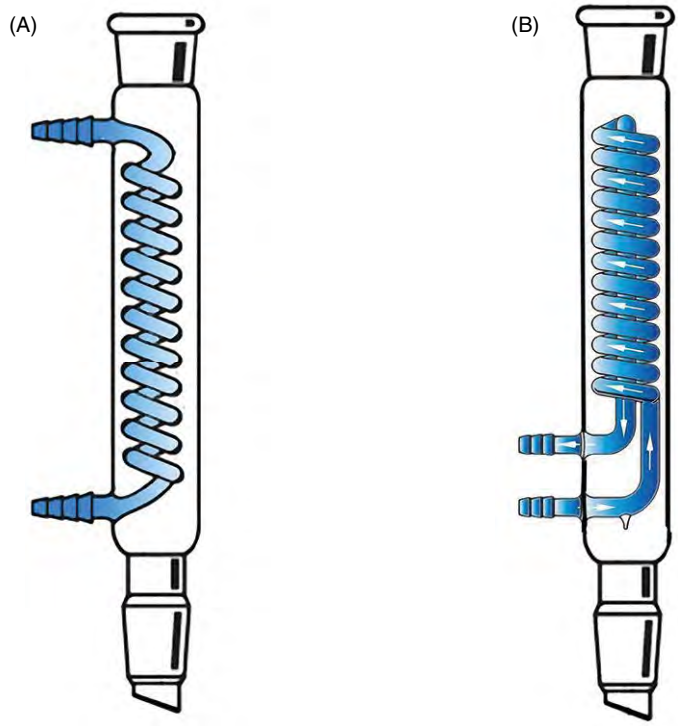

Fig. 10.2. A glass condenser is used during the boiling of the propionic-orcein stain to recover the vapours that are being boiled away. **(A)** Graham condenser. **(B)** Dimroth condenser. Also, glass beads or boiling stones are used in the stain solution to prevent rapid and dangerous boiling.

0.9% NaCl or, preferably, KCl. Alternatively, small pieces of galled roots can be placed into the same solution in a Syracuse watch glass and the females can be extracted directly into the solution. Never attempt to extract females in distilled water; they usually burst in a few minutes because of high osmotic turgor pressure. Extracted females should be used for cytological preparations immediately, or within an hour after extraction from the roots.

10.2.2 Preparation of smears to study oogonial divisions and early stages of maturation of oocytes

A video of this technique is available for viewing at the following URL: https://www.researchgate.net/publication/316846251_Staining_Chromosomes_of_the_root-knot_nematodes_Meloidogyne_species.

Using a fine pipette, draw four of the females extracted early from the galled roots and transfer them with a small drop of tap water onto a clean slide (rinsing with alcohol and wiping with tissue paper is sufficient). With very fine forceps, remove one female from the drop, and deposit it in the centre of the same slide with as little water as possible. Wait for a few seconds until all the water that surrounds the female has evaporated. Then, holding the female with the forceps, make a cut at the neck region with a sharp, surgical eye-knife. Immediately draw the female body with the forceps along the slide, applying slight pressure so that the body contents are smeared uniformly along a strip about 0.5 to 1.0 cm long. The smear dries in a few seconds and becomes clearly visible as a white strip. Smear the other three females in parallel strips, spaced about 2 mm from each other. Proceed immediately with the hydrolysis of the material.

10.2.2.1 Hydrolysis

Time = 5–10 min. Immerse the slide with the smears in a 1 M HCl solution prepared by mixing about 10 ml of reagent HCl in 100 ml distilled water (this should be done immediately after you have made the smears, i.e. before the nematode material has dried excessively). Some material may be lost from the surface of the slides during hydrolysis. To minimize the loss of material, invert the slide and then lower it into the dish containing the HCl solution. The nematode material will thus be pressed against the slide as it touches the surface of the HCl solution and will not be washed sideways. If, in spite of these precautions, much material is still lost in a later step, allow the smears to dry for a longer period, i.e. 3 to 5 min, before immersing the slide into the HCl. In fact, the smears can be prepared and kept for several days, and possibly months, in a dry environment before they are processed for staining, with only slight reduction in quality of the final preparations. For best results, hydrolysis should be done immediately without drying the material excessively. Leave the slide in the HCl solution for about 5–10 min at room temperature, then remove it and wipe dry with tissue paper, leaving wet only the smears and a small area surrounding them.

10.2.2.2 Fixation

Time = 20–30 min or 40–60 min. The slide is now ready and should be immersed in the fixative before the smears dry out completely. It is important that most of the water is removed from the slide before immersion into the fixative because the fixative itself contains no water and should be kept free from water contamination. Immerse the slide in a Coplin staining dish or other glass dish filled with freshly prepared fixative consisting of three parts of absolute ethyl alcohol + one part glacial acetic acid. About 20 to 30 min of fixation are sufficient for oogonia and young oocytes at metaphase I, but 40 to 60 min may be required for oocytes at anaphase I, telophase I, or more advanced stages of maturation. During fixation, the nematode material turns white, and the smears are easily visible on the slide.

10.2.2.3 Staining

Time = 20–40 min. Remove the slide from the fixative and wipe it dry with tissue paper, leaving wet only the smears and a small area surrounding them. Place the slide on a perfectly level surface and wait a few seconds, until much of the fixative has evaporated from the area surrounding the smears. The smears themselves should be still wet. Apply one or two drops of orcein stain on the smears. Cover the stain with a deep-well (cavity) slide to prevent evaporation of the acid and precipitation of stain particles. For best results, place the slide on a piece of plate glass previously levelled perfectly on a working table. If the slide is not perfectly level, the stain will have a tendency to move away from the smears and be sucked between the two slides. The same will happen if too much stain has been applied on the smears because the drop of stain spreads over a larger area as soon as it is covered with the cavity slide.

10.2.2.4 Mounting

Remove the cavity slide and hold the slide with the smears vertically on a piece of tissue paper to drain the excess stain. The smears will still be soaked with stain. To remove the rest of the stain, immerse the slide for 3 or 5 s in a glass dish containing 45% propionic acid. Remove the slide from the 45% propionic acid and place it on absorbent paper on the working table. The upper side of the slide that carries the smears will still be wet. Take with the forceps a No. 1, 22 mm square cover slip, dip it momentarily in the staining dish with the 45% propionic acid to wet it, then apply it on the wet smears. Absorb the excess acid solution with tissue paper until the cover slip settles firmly over the smears and does not move sideways. (Any side movement of the cover slip should be avoided because the soft and delicate oocytes are easily destroyed.) Allow the slide to dry slowly for 5 to 10 min. Seal the mount as soon as some air starts moving under the edges of the cover slip. A sealing medium can be prepared as follows: (i) add equal weights of paraffin and lanolin in a beaker; (ii) place it in a hot water bath until the paraffin melts; (iii)

put the liquefied mixture into small flat cardboard containers, e.g. slide boxes, and allow to cool and solidify. A wire that is approximately 2 to 3 mm thick, 10 mm long, and attached to a wooden holder makes a suitable sealing applicator. Heat the wire end of the applicator over the flame from an alcohol burner, and then touch the paraffin–lanolin mixture for a few seconds to produce a small well of melted sealing material. The applicator may have to be reheated several times to ensure that the sealing medium is hot when deposited on the slide. If the medium is not sufficiently hot, the bonding with the glass surfaces will not be strong, and air may move under the cover slip within a few days. When application is performed properly, the mount may not dry out even after 6 months. The slide thus prepared is a temporary mount, ready for microscopic examination.

10.2.2.5 Examination of temporary preparations

The temporary preparations should be examined microscopically within 3 or 4 days (Figs 10.3 and 10.4). During this period, the oogonia and oocytes, as well as the rest of the nematode material surrounding them remain soft. If further spreading of the chromosomes is desired, gentle pressure can be applied locally on the cover slip with a needle under low (100×) magnification. The material will harden within 4 to 5 days and pressure is no longer effective in spreading the chromosomes and will cause cracking, thus damaging the preparations. Still, such older preparations can be in satisfactory condition for cytological observations for several weeks, and even 2 to 3 months, if the mount is sealed properly so that it will not dry out.

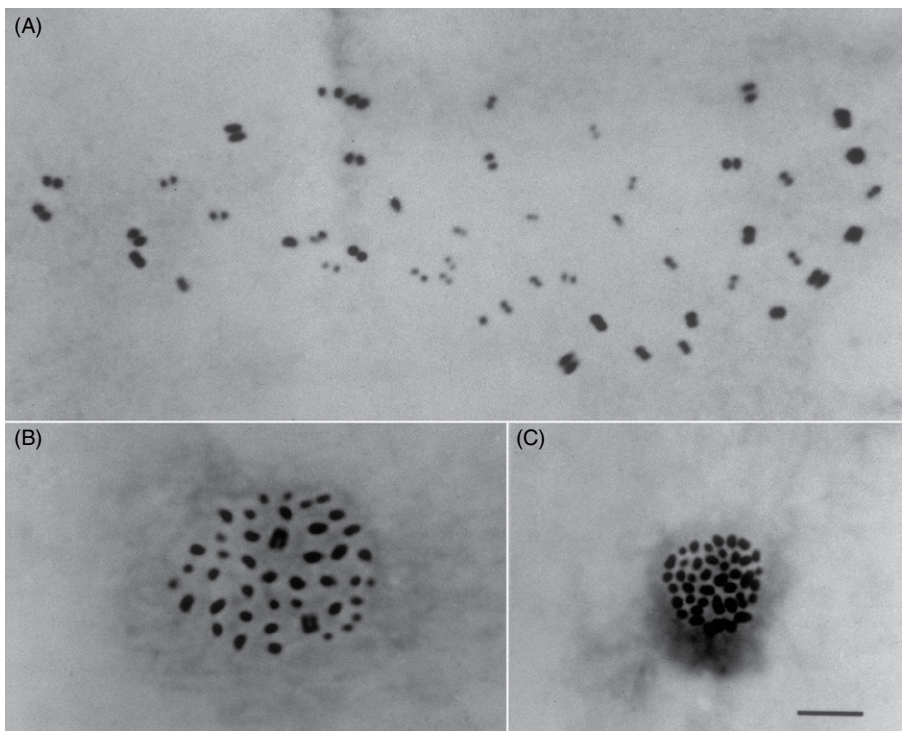

Fig. 10.3. **(A)** Photomicrograph of 48 univalent chromosomes (dyads) of *Meloidogyne javanica* at pro-metaphase of the first and only maturation division. (After Triantaphyllou, 1979.) **(B)** Metaphase chromosomes of *M. incognita* showing how the chromosomes are tightly compressed but can be spread apart with slight pressure on the cover slip. **(C)** Telophase chromosomes of *M. javanica*. Scale bar = 3 µm. (After Triantaphyllou, 1985b.)

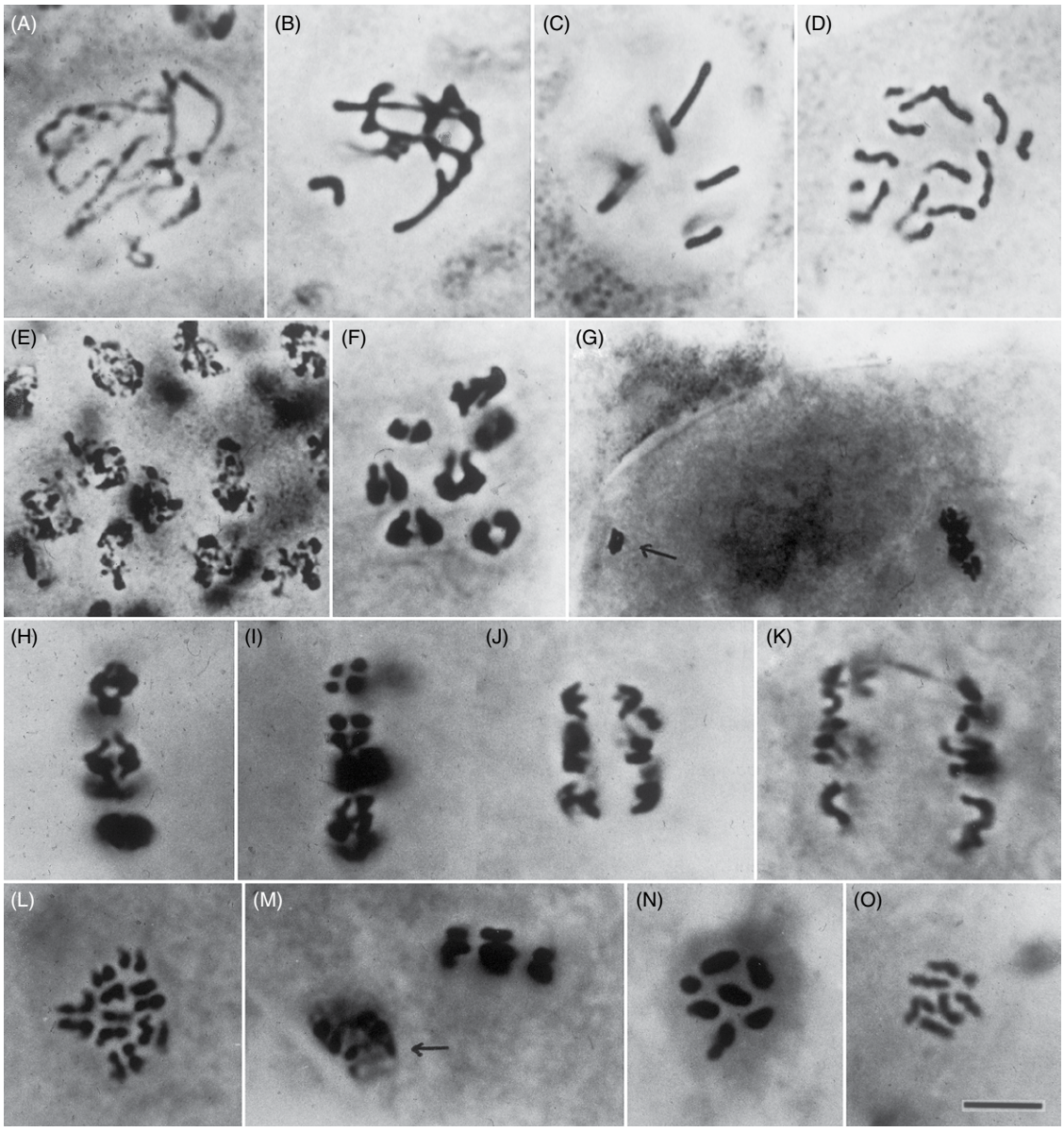

Fig. 10.4. Photomicrographs of chromosomes *Meloidogyne kikuyensis* in various stages of oogenesis. **(A)** Early prophase. **(B)** Mid-prophase with interconnected, condensed chromosomes. **(C)** Late prophase. **(D)** Early metaphase. **(E)** Zone of synapsis. **(F)** Seven diakinetic chromosomes shortly before it enters the oviduct/spermatotheca. **(G)** Metaphase-I of an oocyte and a sperm nucleus (arrow). **(H)** Typical metaphase-I. **(I)** As in Fig. 10.3B but, as typical for *Meloidogyne*, with two bivalents that form perfect tetrads. **(J, K)** Advanced anaphase-I figures of complex chromosomes, but not perfect dyads, with some chromosomal bridges. **(L)** Telophase-I in polar view. **(M)** Metaphase-II and the first polar body (arrow). **(N)** Metaphase-II. **(O)** Telophase-II in polar view, with six long and one short chromosome. Scale bar = 3 µm. (After Triantaphyllou, 1990.)

10.2.3 Summary of procedures for staining *Meloidogyne* chromosomes with propionic-orcein

A. Preparation of material

- Place healthy, whole, pearly white females from 45-day-old cultures into a drop of phosphate buffered saline on a glass slide.
- Dissect the females to release the gonads.
- Remove the body.
- Spread the gonads in a smear on the glass slide.
- Air dry the smear to make it adhere to the slide.

B. Hydrolysis

- Immerse the slides into 1 M HCl for 20–30 min at room temperature (10 ml of reagent HCl + 100 ml distilled water).
- Remove and air dry before fixation.

C. Fixation

- Immerse the slides into a 3:1 solution of absolute ethanol and glacial acetic acid for 20–60 min.
- Remove and air dry.

D. Staining (20–40 min)

- Place 2 ml of stain onto tissues for 20–40 min. Stain:
  - 2.2 g orcein stain (natural or synthetic) + 100 ml glacial propionic acid
  - 100 ml distilled water
  - fine paper filter
  - dark brown glass bottles ground glass stopper and pipette.

E. Mounting (3 to 5 s for each step)

- Wash slides in 45% propionic acid.
- Add a propionic soaked cover slip (No.1 22 mm square).
- Seal with glyceel, fingernail polish, or paraffin/lanolin mixture (equal weights of paraffin and lanolin).
- A sealing tool: a 2–3 mm thick copper wire slightly curved at the end, 10 mm long, and attached to a wooden handle.

F. Observation

- Use an oil immersion objective and a green filter to increase contrast and enhance definition.
- Photograph with a digital camera.

10.3 Staining Chromosomes of Free-living Nematodes

10.3.1 Propionic-orcein staining (Hechler, 1970)

- Kill and fix adult nematodes for 25 min or longer in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid).
- Prepare aceto-orcein stain:
  - boil 55 ml glacial acetic acid
  - pour over 1 g of orcein powder
  - cool to room temperature and add 45 ml of distilled water

- filter through Whatman No. 1 filter
- stain for 12 h or longer
- gentle pressure on the cover slip may flatten certain nuclei for a better presentation.

- Add a cover slip and seal with nail polish or other sealer.

10.3.2 DAPI fluorescent nuclear staining (Hasegawa *et al.*, 2006)

- Place adult nematodes into a drop of 0.1 M NaCl into the well of a glass cavity slide.
- Cut the anterior end of the nematode to release the germ cells.
- Exchange the NaCl with –20°C acetone with a pipette.
- Air dry the specimen.
- Stain with 2 µg ml^{-1} of DAPI in phosphate buffered saline (PBS) for 5 min:
  - 137 mM NaCl
  - 8.1 mM Na_2HPO_4
  - 2.68 mM KCl
  - 1.47 mM KH_2PO_4.
- Wash stained cells with PBS for several seconds.
- Mount in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA).
- Observe with a fluorescent or confocal laser-scanning microscope.

10.4 Staining Chromosomes of Giant Cells

Plant-parasitic nematodes, especially the sedentary endoparasites, take over the cell cycle of the plant and cause it to produce very specialized cells as their feeding sites (Vieira and Engler, 2015). In the root-knot nematodes, these feeding sites are formed by repeated mitosis without cytokinesis so that they contain multiple copies of somatic chromosomes. Root-knot nematodes interfere with the plant cell cycle and thereby alter the chromosome number and morphology of the feeding sites. Staining chromosomes of these cells is useful for studying the complex relationship between the nematode and its host as part of the search for tactics to reduce the damage that nematodes cause. The following techniques have been useful in staining the chromosomes of nematode feeding sites (Carter *et al.*, 1977; Price *et al.*, 1980; Wiggers, *et al.*, 1990).

10.4.1 Seed germination pouch cultures

- Grow plants in seed germination pouches (Fig. 10.5) (Atamian *et al.*, 2012) at 24°C day/ 20°C night temperatures and 12 h light/dark cycle.
- Inoculate with 75–100 freshly hatched second-stage juveniles of *Meloidogyne* per 6-day-old seedling.
- Grow plants until nematode egg masses appear on the galled root surfaces, 4–6 weeks after inoculation.
- Cut infected root sections and fix in 3:1 ethanol/glacial acetic acid for 24 h.
- Store in 70% ethanol for several months until stain is applied.

10.4.2 Glasshouse cultures

- Grow tomato seedling in a 1:1 sand:peat mix in a glasshouse at 22–30°C.
- Inoculate each 6-week-old seedling with 5000 eggs of root-knot second-stage juveniles.
- Grow plants until nematode egg masses appear on the galled root surfaces, 4–6 weeks after inoculation.

Fig. 10.5. Seed germination pouch with seedlings used to culture root-knot and cyst nematodes (https://mega-international.com) (Atamian *et al*., 2012).

- Cut infected root sections and fix in 3:1 ethanol/glacial acetic acid for 24 h.
- Store in 70% ethanol for several months until stain is applied.

10.4.3 Feulgen staining

- Hydrolyse fixed specimens in 5 N HCl for 40 min at 25°C.
- Prepare Schiff's reagent by dissolving 2 g of basic fuchsin in 400 ml of boiling water.
- Cool to room temperature and add 4 g of potassium metabisulphite ($K_2S_2O_5$) and 40 ml of 1 N HCl.
- Store overnight in a dark bottle.
- Add 1 g of Norit A decolorizing charcoal and shake.
- Vacuum filter through a Whatman No. 1 filter paper producing a clear stain.
- Stain material in Schiff's reagent for 2 h.
- Rinse twice in SO_2 water (600 ml of H_2O, 36 ml of 1% $K_2S_2O_5$, and 30 ml of 1 N HCl) for 10 min each.
- Rinse in distilled water for 10 min.

10.4.4 Observation of stained material

- Cut galls out of stained tissues.
- Tease giant cells out of tissues with an eye-knife and sharply pointed forceps.
- Place giant cells into a drop of 45% lactic acid on a microscope slide.
- Place a cover slip on the giant cells and squash them.
- Immerse in liquid nitrogen, remove the cover slip with a scalpel and dry overnight.
- Apply a small drop of Permount™ on top of giant cells and cover with a cover slip.
- Observe with a compound microscope fitted with an oil immersion lens at 1000× magnification (Fig. 10.6).

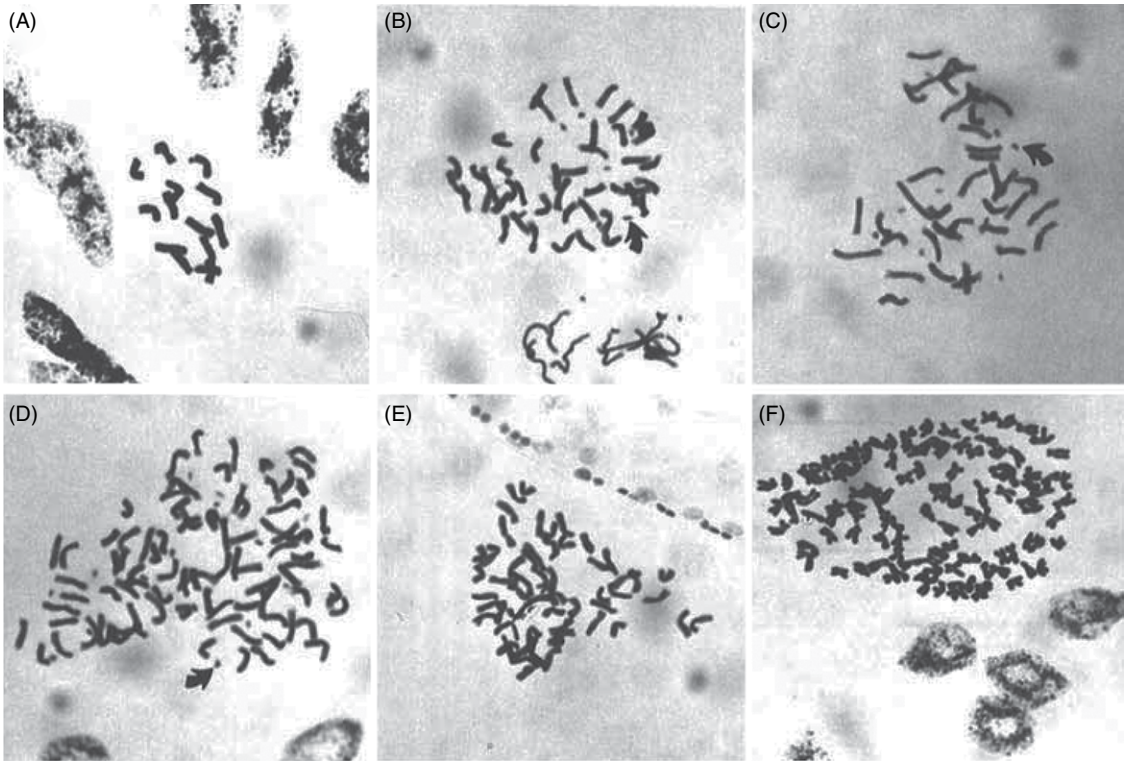

Fig. 10.6. Chromosomes of giant cells of pea (*Pisum sativum* 'Little Marvel') stained with Feulgen, protocol of Price *et al*. (1980) as used by Wiggers *et al*. (1990). (A) 2n = 14; (B) n = 45; (C) n = 37; (D) n = 88; (E) n = 45; (F) n = approximately 112. (Figure from Wiggers *et al*. (1990), with permission.)

10.5 References

Atamian, H.S., Roberts, P.A. and I. Kolshian. (2012) High and low throughput screens with root-knot nematodes *Meloidogyne* spp. *Journal of Visualized Experiments* 61, e3629. DOI: 10.3791/3629

Grisi, E., Burrows, P.R., Perry, R.N. and Hominick, W.M. (1995) The genome size and chromosome compliment of the potato cyst nematode *Globodera pallida*. *Fundamental and Applied Nematology* 18, 67–70.

Hasegawa, K., Mota, M.M., Futai, K. and Miwa, J. (2006) Chromosome structure and behaviour in *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae) germ cells and early embryo. *Nematology* 8, 425–434. DOI: 10.1163/156854106778493475

Hechler, H.C. (1970) Chromosome number and reproduction in *Mononchoides changi* (Nematoda: Diplogasterinae). *Journal of Nematology* 2, 125–130.

Price, H.J., Bachmann, K., Chambers, K.L. and Riggs, J. (1980) Detection of intraspecific variation in nuclear DNA content in *Microseris douglasii*. *Botanical Gazette* 141,195–198. DOI: 10.1086/337144

Sobczak M. and Golinowski W. (2009) Structure of cyst nematode feeding sites. In: Berg R.H. and Taylor C.G. (eds) *Cell Biology of Plant Nematode Parasitism. Plant Cell Monographs*, *Vol. 15*. Springer-Verlag, Berlin, Germany. DOI: 10.1007/7089_2008-_38

Triantaphyllou, A.C. (1979) Cytogentics of root-knot nematodes. In: Lamberti, F. and Taylor, C.E. (eds) *Root-knot nematodes* (Meloidogyne *species*). Academic Press, New York, New York, USA, pp. 85–109.

Triantaphyllou, A.C. (1981) Oogenesis and the chromosomes of the parthenogenic root-knot nematode *Meloidogyne incognita. Journal of Nematology* 13, 95–104.

Triantaphyllou, A.C. (1985a) Cytogenetics, cytotaxonomy, and phylogeny of root-knot nematodes. In: J.N. Sasser and Carter, C.C. (eds) *An Advanced Treatise on* Meloidogyne*, Vol. 1. Biology and Control*. North Carolina State University Graphics, Raleigh, North Carolina, USA, pp. 111–133.

Triantaphyllou, A.C. (1985b) Cytological methods for the study of oogenesis and reproduction of root-knot nematodes. In: Barker, K.R., Carter, C.C. and Sasser, J.N. (eds) *An Advanced Treatise on* Meloidogyne*, Vol. 2*. *Methodology*. North Carolina State University Graphics, Raleigh, North Carolina, USA, pp. 107–114.

Triantaphyllou, A.C. (1990) Cytogenetic status of *Meloidogyne kikuyensis* is relation to other root-knot nematodes. *Revue de Nématologie* 13, 175–180.

Vieira, P. and Engler, J.de A. (2015) The plant cell inhibitor KRP6 is involved in multinucleation and cytokinesis disruption in giant-feeding cells induced by root-knot nematodes. *Plant Signaling and Behavior* 10, e1010924. DOI: 10.1080/15592324.2015.1010924

Wiggers, R.J., Starr, J.L. and Price, H.J. (1990) DNA content and variation in chromosome number in plant cells affected by *Meloidogyne incognita* and *M. arenaria. Phytopathology* 80, 1391–1395. DOI: 10.1094/Phyto-80-1391

Yoshida, K., Hasegawa, K., Mochiji, N. and Miwa, J. (2009) Early embryogenesis and anterior-posterior axis formation in the white-tip nematode *Aphelenchoides besseyi* (Nematoda: Aphelenchoididae). *Journal of Nematology* 41, 17–22.

11 Isoelectric Focusing of Proteins

JANETE A. BRITO

Florida Department of Agriculture and Consumer Services, Gainesville, Florida, USA

11.1 Introduction

Electrophoresis is used to separate charged molecules according to size. Charged molecules, like DNA, RNA or proteins, move through a gel when an electric current is passed across it, with one end of the gel having a negative charge and the other a positive charge. Molecules migrate in the gel according to charge; positively charged molecules will move to the negative end, whereas negatively charged molecules like DNA will move towards the positive end. Small molecules migrate more quickly and move further than larger molecules, which travel a relatively shorter distance. Thus, the molecules are separated by size. Isoelectric focusing (IEF), also termed electrofocusing, is a technique for separating charged molecules by differences in their isoelectric point (pI). In an electric field, molecules in a pH gradient in a gel will migrate towards their pI, which is the pH at which the molecule has no charge. The concentrating effects of this technique leads to the name 'focusing', making it extremely useful to work with small number of enzymes and proteins.

The first study demonstrating the usefulness of biochemical markers resolved by electrophoresis for the identification of plant-parasitic nematodes was carried out by Dickson *et al.* (1971). These authors showed that some protein band patterns, when visualized using a protein-specific stain, were valuable for differentiating certain species of *Meloidogyne* and *Ditylenchus*, as well as in the genera *Heterodera* and *Aphelenchus*. This electrophoresis study was followed by a report showing distinct differences in soluble protein and some enzyme patterns between *Ditylenchus dipsaci* and *D. triformis* (Hussey and Krusberg, 1971), and a confirmation of distinct protein patterns existing between *Meloidogyne javanica* and *M. arenaria* (Hussey *et al*., 1972). Subsequently, a major protein band differentiating two populations of *Radopholus similis* was reported (Huettel *et al*., 1983), and distinguishable protein patterns were found among *Heterodera* spp. (Pozdol and Noel, 1984) as well as *Globodera rostochiensis* and *G. pallida* (Bakker *et al*., 1988). Following these pioneering studies, it was reported that isoelectric focusing (IEF) could provide a better separation of total soluble protein extracted from some microorganisms, including plant-parasitic nematodes (Fleming and Marks, 1983; Wharton *et al*., 1983; Fox and Atkinson, 1984; Lawson *et al*., 1984). This method allowed a distinct electrophoretic protein profile from extracts of a single nematode specimen. This was an important step as it provided a method for determining intraspecific variation within a single species.

The use of IEF to examine general proteins and specific enzymatic differences among plant-parasitic nematodes, particularly the potato cyst nematodes, *G. pallida* and *G. rostochiensis*, was reported in 1983 (Fleming and Marks, 1983; Wharton *et al*., 1983). Similarly, Fox and Atkinson (1984) observed variations in the protein patterns among pathotypes of these nematode species, particularly the presence of a major band at pH 8.0 in all five pathotypes of *G. rostochiensis*. This band was not detected in *G. pallida*. Additionally, Radice *et al*. (1988) used female extracts obtained from *Heterodera glycines* and *H. avenae* complex to determine genetic variation among these species and isolates using IEF to resolve proteins and isozymes. Late in the 1980s an automated electrophoresis system (Pharmacia PhastSystem®; Uppsala, Sweden; Fig. 11.1)

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
10.1079/9781786391759.0011

Fig. 11.1. Pharmacia PhastSystem electrophoresis system, LKB Pharmacia.

became available making the routine work on enzymes for the diagnostic of plant-parasitic nematodes much more convenient, simpler and safer (Esbenshade and Triantaphyllou, 1990). Karssen *et al.* (1995) adapted the original method for differentiating *G. rostochiensis* and *G. pallida*, described by Fleming and Marks (1983), and root-knot nematodes species for the PhastSystem, which is still being used today.

11.2 Isoelectric Focusing of Proteins for Diagnostics of Cyst Nematodes

The following protocol for use with cyst nematodes was originally described by Fleming and Marks (1983) with modifications by Karssen *et al.* (1995), using the automated electrophoresis PhastSystem mentioned above. This equipment is in use in many laboratories worldwide. It combines automatic sample application, electrophoretic separation and staining in one system, and provides rapid separation and staining. The separation and control unit houses the stainless-steel separation bed and the programmable power supply. The bed can process two gels simultaneously, providing a capacity of up to 24 samples, and temperature can be maintained in the range 4 to 30°C. In addition to temperature, controlled parameters include voltage, current, power and volt-hours. The method described below is for cyst nematodes; one or several cysts filled with eggs and juveniles are required for this identification.

11.2.1 Sample preparation and loading

- Air-dried cysts are pre-soaked in 1% glycerin for at least 12 h at room temperature (21°C).
- Place PhastGel sample-well stamp on ice and use for protein extraction.
- Add one pre-soaked cyst to each of the 12 small sample wells containing 0.7 µl of 1% glycerin.
- Crush and macerate cysts with a small glass rod for 15 s per well to release egg contents.
- Use Pharmacia broad pI calibration kit with pI gradient 3.5–9.3 as reference and pI measuring. Add marker (0.7 µl) to one or two middle wells.
- Load homogenates from wells on the 12/0.3 sample applicator (0.3 µl sample per well) and insert in the middle position of the apparatus applicator arm.

11.2.2 Isoelectric focusing run

IEF is run with PhastGel IEF 3–9 or 5–8 with pH gradients of 3–9 and 5–8, respectively. The following standard programs are used:

- PhastGel IEF 3–9

Sample application down at	1.2	0 Vh		
Sample application up at	1.3	0 Vh		
Separation 1.1 2000 V	2.5 mA	3.5 W	15°C	75 Vh
Separation 1.2 200 V	2.5 mA	3.5 W	15°C	15 Vh
Separation 1.3 2000 V	2.5 mA	3.5 W	15°C	410 Vh

- PhastGel IEF 5–8

Sample application down at	2.2	0 Vh		
Sample application up at	2.3	0 Vh		
Separation 2.1 2000 V	2.0 mA	3.5 W	15°C	75 Vh
Separation 2.2 200 V	2.0 mA	3.5 W	15°C	15 Vh
Separation 2.3 2000 V	2.0 mA	3.5 W	15°C	510 Vh

Total electrophoresis time is approximately 30 min. For more specific information on IEF running conditions see Pharmacia separation technique, file No. 100.

11.2.3 Gel staining

Stain gels with PhastGel Silver Kit according to the instruction manual, with the following modifications (Karssen *et al.*, 1995). The background reducer step (Table 1 of the manual, program #14) was changed from 1.5 min to 1.3 min and the Tris-HCl was replaced with 5 µl acetic acid.

11.2.4 Interpretation of results

Substantial and valuable differences in the protein profiles were observed (Karssen, 1995) between the two *Globodera* spp. (Fig. 11.2). *Globodera pallida* shows one stable major band at pI 5.9 (A) and unique weaker band at pI 6.9 (B). For *G. rostochiensis* one major stable band was observed at pI 5.7 (C) and a weaker band at pI 8.7 (D), which is absent in the *G. pallida* pattern. The pH range of 5–8 proved to be more useful for species identification and differentiation. In another study, a single cyst of *G. tabacum* showed a major band at pI 8.5, whereas *G. artemisiae* showed two major bands between pI 5.85 and pI 5.8 (Sumiya *et al.*, 2002).

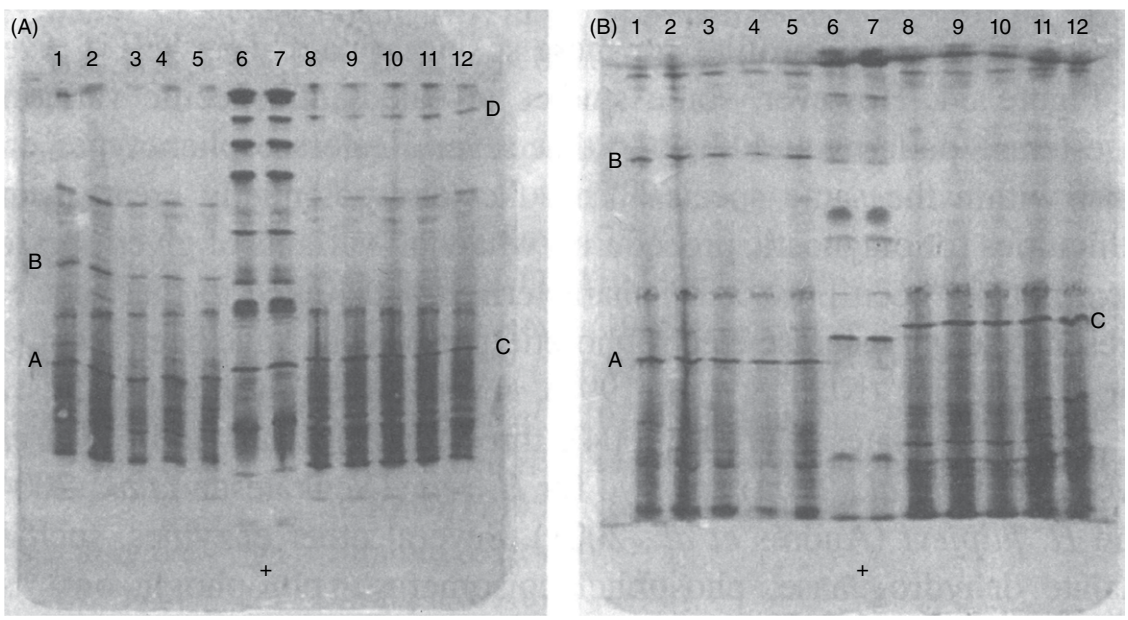

Fig. 11.2. Isoelectric focusing (IEF) patterns of *Globodera pallida* (lanes 1, 2, 3, 4, 5) and *G. rostochiensis* (8, 9, 10, 11, 12) individual specimens and broad pI callibration kit (lanes 6 and 7). **(A)** IEF on PhastGel 3–9. **(B)** IEF on PhastGel 5-8, followed by silver staining. Specific bands are marked by capital letters on the gel. (After Karssen *et al.*, 1995.)

11.3 Isoelectric Focusing of Isozymes for Diagnostics of Root-knot Nematodes

Isozymes (or isoenzymes) catalyse the same chemical reaction but differ in amino acid sequence, which provides a change in electrical charge and, thus, kinetic properties. This enables isozymes to be distinguished from each other by electrophoretic mobility and such differences have been examined for diagnostic purposes using isolectric focusing. For example, isozymes were used to separate species of *Meloidogyne* (Dickson *et al.*, 1971; Hussey *et al.*, 1972; Dalmasso and Bergé, 1978; Esbenshade and Triantaphyllou, 1990; Ibrahim and Perry, 1993), *Ditylenchus* (Dickson *et al.*, 1971) and *Heterodera* (Nobbs *et al.*, 1992; Ibrahim and Rowe, 1995). Ibrahim *et al.* (1995) examined the isoenzyme phenotypes of esterase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, phosphoglucose isomerase and phosphoglucomutase in six species of *Pratylenchus* and the dendrogram from the enzyme banding patterns gave two groups: group 1 contained *P. pinguicaudatus*, *P. fallax* and *P. thornei* and group 2 contained *P. penetrans*, *P. neglectus* and *P. crenatus*. Ibrahim *et al.* (1996) showed that a monoclonal antibody to a diagnostic non-specific esterase discriminated *M. incognita* from *M. javanica* without the need to separate the esterases by electrophoresis first. Although the use of isozymes for chemotaxonomy has largely been superseded by molecular techniques, the approach is still useful to differentiate species of *Meloidogyne* and can be used with a single egg-laying female (see below) and with galled roots (Ibrahim and Perry, 1993).

Esbenshade and Triantaphyllou (1985a) provided one of the most detailed protocols for nematode sample preparations, isozyme extraction and separation of proteins extracted from young egg-laying females of *Meloidogyne* spp., for diagnostic and nematode species separations as well as protein staining. Cetintas *et al.* (2003) and Brito *et al.* (2008) modified the protocols to use with an inexpensive and simple electrophoresis system (Mini-Protean® III cell, BioRad) (see Fig. 11.3). This apparatus may be used with both commercially available, ready-to use precast or laboratory prepared gels; the protocols described and discussed below apply to a discontinuous native polyacrylamide gel electrophoresis (PAGE).

11.3.1 Preparation of egg-laying females for isozyme extraction

- Wash the root sample thoroughly to remove soil and other organic matter.
- Add 5 µl deionized water (DI) into a 0.6 ml microfuge tube and an equal volume (5 µl) of Native sample buffer (2×) in each of the tubes.
- Place a single *Meloidogyne* female (unknown species) in each 0.6 ml microfuge tube. Keep the microfuge tubes containing the females on ice. Pick females from different parts of the root systems to increase the chance of finding mixed species, if present. Make sure that females are free of plant tissue and soil debris.
- Use nematodes immediately or freeze samples at –20°C for up to 3 months.

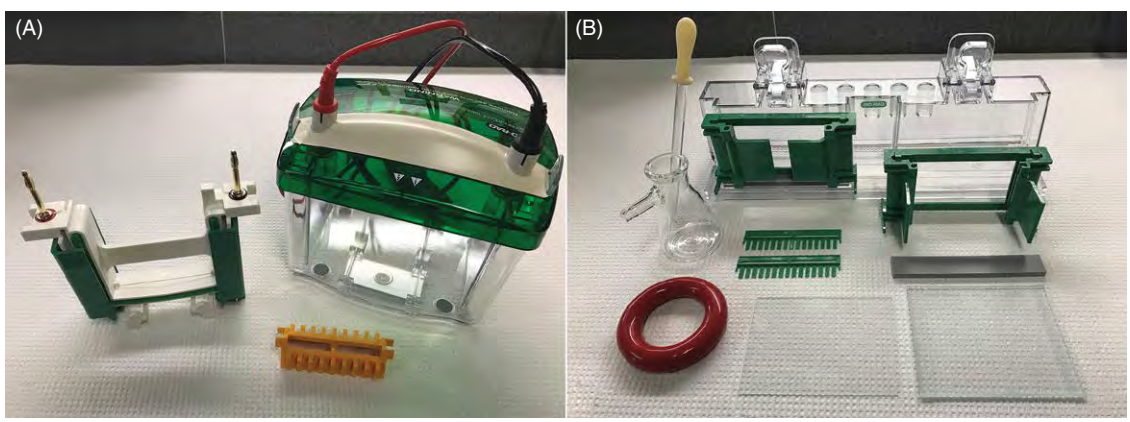

Fig. 11.3. Mini-Protean Cell System (BioRad). **(A)** Electrophoresis apparatus, electrode assembly and sample loading guide. **(B)** Casting stand, casting frame, glass plates, plastic combs, micro filtering flask with sidearm tabulation, and a lead flask ring.

11.3.2 Gel preparation

11.3.2.1 Resolving gel (8%); reagents and preparation

2.7 ml Acrylamide / Bis (30% T[total monomer concentration, 2.67%] C [crosslinking monomer concentration]) (**stock solution**)
29.2 g Acrylamide
0.8 g N,N'-Methylene-bis-acrylamide

- Bring volume up to 100 ml distilled water (dH_2O). Keep it in the dark in a refrigerator for up to 3 months.

2.5 ml 1.5 M TRIS-HCl, pH 8.8 (stock solution)
18.15 g TRIS BASE
60 ml dH_2O

- Adjust pH to 8.8 with 6 N HCl
- Bring volume up to 100 ml with dH_2O. Keep it in the refrigerator up to 3 months.

50 µl 10% APS (ammonium persulfate) fresh daily

- Use 100 mg APS for 1ml dH_20

5 µl TEMED

- Prepare the monomer solution (resolving gel) by combining all reagents except the catalysts (APS and TEMED). Degas the solution for 15 min (Fig. 11.4).
- While the monomer solution is degassing, assemble the electrophoresis apparatus (Fig. 11.3). If using the glass plates with 0.75 mm integrated spacers you must also use 0.75 mm comb.
- Immediately prior to casting the resolving gel, add the two catalysts (APS and TEMED). Swirl gently to initiate polymerization. If using a Pasteur pipette to cast the gel, leave some gel solution within the pipette to monitor the polymerization. The gel level should be 1 cm below the comb teeth.
- Immediately overlay the resolving gel with water-saturated n-butyl alcohol. Allow the gel to polymerize (30–45 min).

Fig. 11.4. Vacuum pump used to remove gas molecules from the acrylamide solution.

11.3.2.2 Stacking gel (4%); reagents and preparation

1.3 ml Acrylamide/Bis
2.5 ml 0.5 M TRIS-HCL, pH 6.8 (stock solution)
6.0 g TRIS BASE
60 ml dH_2O

- Adjust pH to 6.8 with 6 N HCl
- Bring volume up to 100 ml with dH_2O. Keep it in the refrigerator for up to 3 months.

6.2 ml dH_2O
(Total volume, 10 ml)
50 µl 10% APS
10 µl TEMED

- Prepare the stacking gel, but without (APS and TEMED). Degas the solution as above.
- Rinse the **overlay solution** with dH_2O using a squeeze bottle. Gently dry the edges of the casting frame.
- Immediately prior to casting the stacking gel add the two catalysts (APS and TEMED). Swirl gently to initiate polymerization. After casting the gel, immediately insert the desired comb. The thickness of the comb used should be the same as that of spacer glass plate. Cetintas *et al*. (2003) used a 15-well comb (0.75 mm), which is sufficient for 13 unknown root-knot nematode females plus two *M. javanica* females (control) (illustration below). Prepare two gels per root system, so a total of 26 females will be examined for each root system. The number of females per root system can be modified, if needed. The control to the right is one lane inwards to ensure that if there are problems with the gel ends there is at least one control available for comparison.

Mj	x	x	x	x	x	x	x	x	x	x	x	Mj	x

- Wait 30–45 min for polymerization during which the Electrode (running) buffer can be prepared from the stock solution.

11.3.2.3 Electrode (running) buffer

Electrode (running) buffer, pH 8.3 (**stock solution**)
30.3 g TRIS BASE
144.0 g Glycine

- Bring volume up to 1000 ml with dH_2O. There is NO need to adjust the pH.

Running buffer:
40 ml 10× Electrode buffer stock
360 ml dH_2O
Total volume, 400 ml

11.3.3 Running the gel

Gently remove the combs from the polymerized gel and rinse the wells thoroughly with dH_2O and assemble the electrophoresis apparatus. Detailed procedures how to assemble casting stand, frame, electrophoresis module and sample loading are provided in the instruction manual (BioRad) (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/4006157B.pdf).

Fill the inner chamber with running buffer and check for leakage and bubbles. Pour the rest of the running buffer in the Mini Tank (lower buffer chamber). A total of 400 ml of the running buffer will be enough for one run.

11.3.4 Isozyme extraction and gel loading

- Place nematode samples on ice using females just picked from infected roots or those that were frozen (see Section 11.3.1).
- Macerate females using plastic toothpicks (ideal), long pipette tips with closed tips or pestles. Do not heat the samples. Use a separate toothpick for each female. DO NOT use wooden toothpicks, because they can absorb the chemical solutions, or toothpicks treated with antibiotics.
- Load 10 µl of the macerate into each appropriate well. Use one gel loading tip for each nematode macerate to avoid cross contamination between samples. A control, e.g. *M. javanica*, should be included.

11.3.5 Electrophoresis

Place the electrophoresis apparatus in a refrigerator and run at 80 V for 15 min and then at 200 V for 35–38 min, or until the dye front is 1–2 mm from the bottom of the gel. While electrophoresis is running prepare the esterase and malate dehydrogenase staining solutions.

Turn power supply off and remove apparatus from refrigerator. Carefully disassemble the inner chamber in a Pyrex dish. Cut the gels at the tracking die front (line) as it will disappear during the isozyme development (staining); this will facilitate calculation of the relative electrophoretic mobility (Ef) of each enzyme, if needed. The Ef is the ratio of the movement of the band to that of the tracking dye (Esbenshade and Triantaphyllou, 1985a).

11.3.6 Staining

Transfer the gel to a Petri dish and incubate it with the newly prepared reagent solution (50 ml) for the desired isozyme detection (Figs 11.5–11.9). If desired, the same gel can be stained for both MDH (malate dehydrogenase) and EST (esterase) detections (Fig. 11.5). First stain it for MDH, then pour off the staining solution and wash gel briefly 3–4 times with dH_2O, then follow the procedure for EST staining.

- For **EST** detection incubate the gel in the staining solution for 45 min at 37°C in the dark (wrap the Petri dish with aluminium foil), then wash gel with dH_2O to stop the development and place gel in the fixative solution.
- For **MDH** detection incubate gel in the staining solution for 15 min at 37°C in the dark, then wash gel with dH_2O to stop the development.
- For **SOD** (superoxide dismutase) detection incubate gel in the staining solution for 20 min at 37°C in the dark. Remove gel from incubator and place under fluorescent light for 15 min. Bands of SOD activity will appear as clear areas in the gel (Esbenshade and Triantaphyllou, 1985b).
- For **GOT** (glutamate-oxaloacetate transaminase) detection incubate gel in the staining solution for 60 min at 37°C. Bands will appear as blue areas on an orange background (Esbenshade and Triantaphyllou, 1985b).

Examine the gel by placing the Petri dish containing the gel on a light box and record the isozyme phenotype. Esbenshade and Triantaphyllou (1985b) provide a list of phenotype designations and explanations. Fix the gel in **fixative solution** (20% ethanol and 10% glycerin) for at least 30 min at room temperature before drying it, if needed. If using the gel-drying frame (14 × 14 cm), follow the manufacturer's instructions to soak cellophane sheets and assemble sheets and gel sandwich.

Figure 11.4 shows both esterase and malate dehydrogenase phenotypes found in a population of *M. enterolobii* from Florida, USA.

11.3.6.1 Staining solution for esterase

α-Naphthyl acetate (1%) in 50% acetone (**stock solution;** fresh daily)

2.5 ml acetone
2.5 ml dH_2O
0.050 g α-naphthylacetate

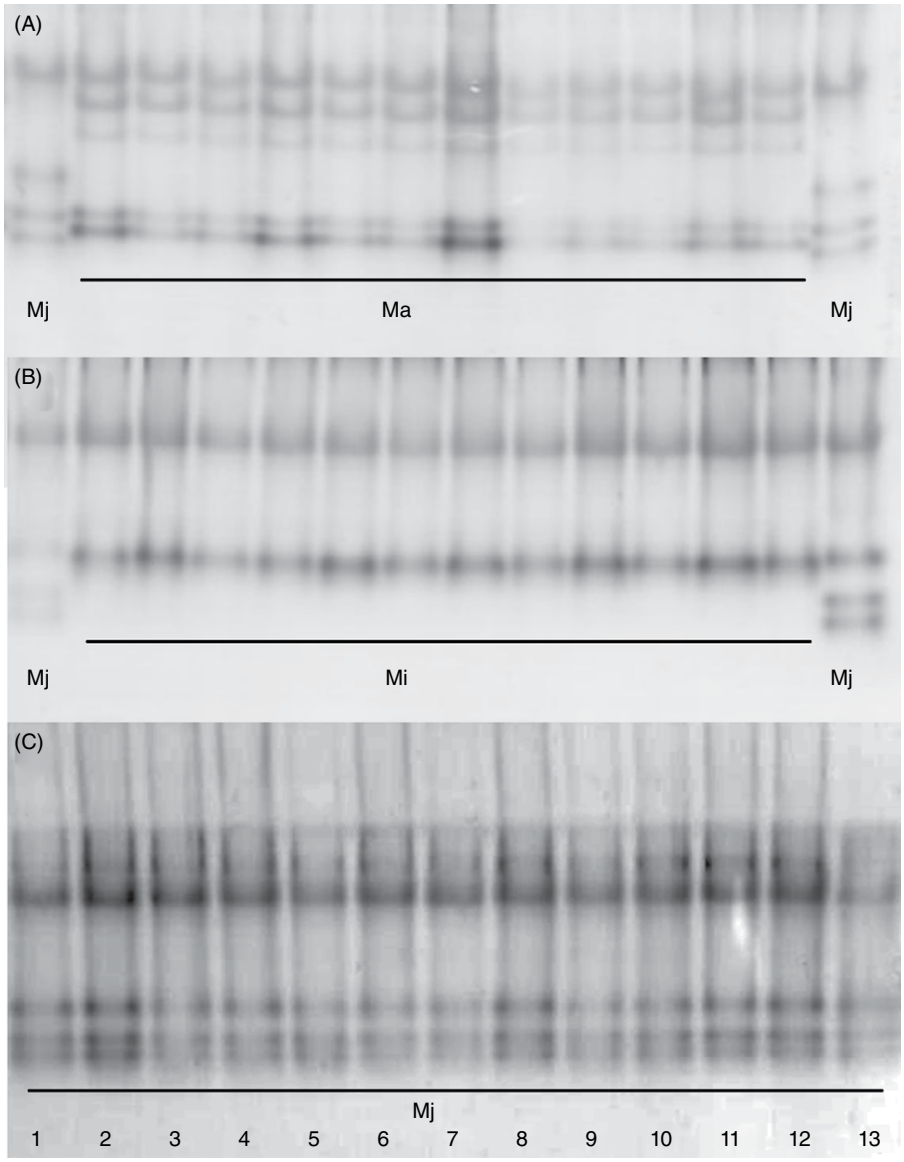

Fig. 11.5. Isozyme phenotypes. Single gels stained for both esterase (EST) (upper bands) and malate dehydrogenase (MDH) (lower bands). **(A)** *Meloidogyne arenaria* (Ma) (lanes 2–13) (A2;N3). **(B)** *M. incognita* (Mi) (I1; N1). **(C)** *M. javanica* (Mj) (J3; N1) phenotypes. *Meloidogyne javanica* was used in each gel as the control.

- Use 3 ml to prepare the esterase staining solution and dispose of the remainder.

0.05 M Potassium phosphate buffer, pH 6.0 (**stock solution**; keep at room temperature).

A: 50 ml of 1 M potassium phosphate, monobasic in 900 ml dH_2O. Adjust pH to 6.0 with 1 M potassium hydroxide. Bring volume up to 1000 ml dH_2O.

B: 25 ml of 1 M potassium phosphate, dibasic in 400 ml dH_2O. Adjust pH to 6.0 with HCl. Bring volume up to 500 ml dH_2O.

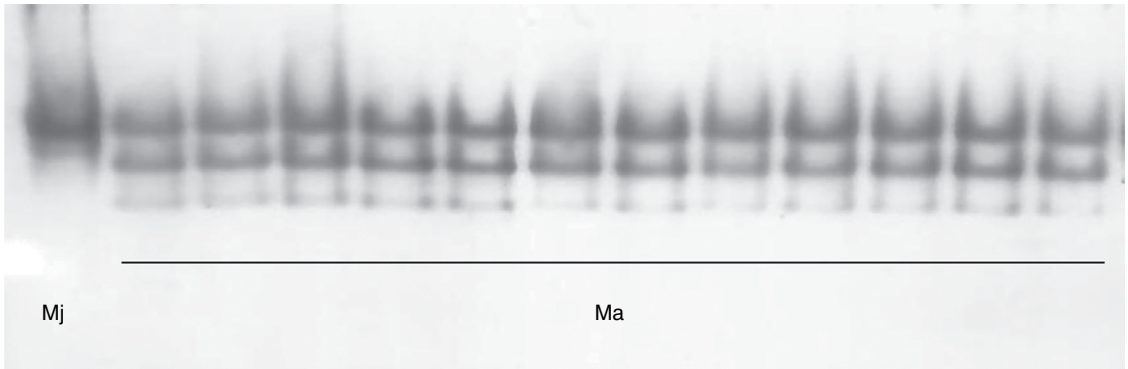

Fig. 11.6. Gel stained only for malate dehydrogenase detection of *Meloidogyne arenaria* (Ma) (N3) phenotype. *Meloidogyne javanica* (Mj) (N1) was used as the control.

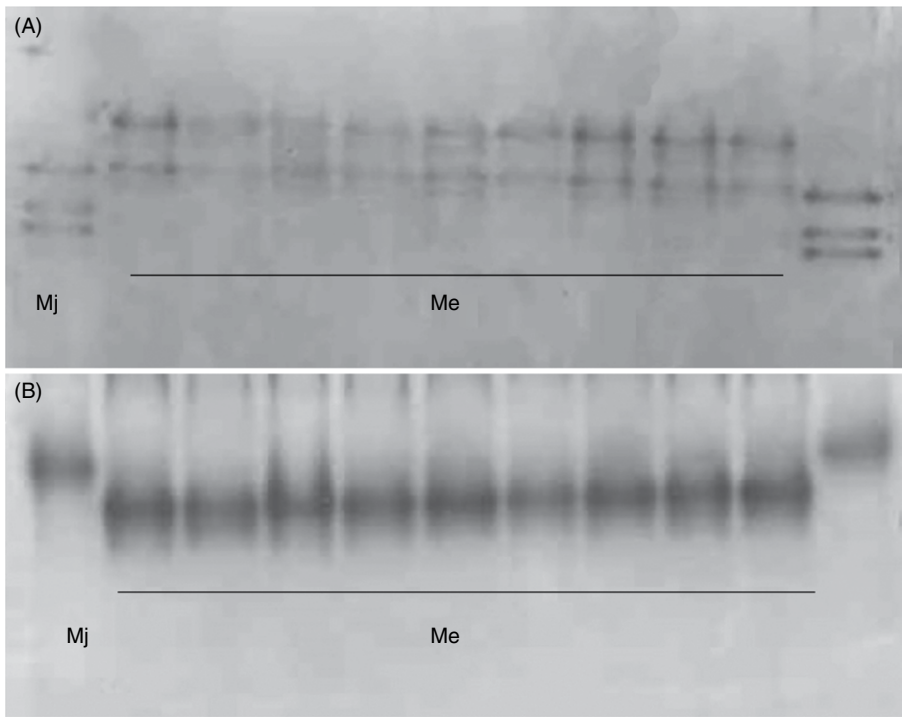

Fig. 11.7. Isozyme phenotypes. **(A)** Gels stained only for esterase detection of *Meloidogyne enterolobii* (Me) (VS1–S1). **(B)** Gel stained only for malate dehydrogenase detection of *M. enterolobii* (Me) (N1a). *Meloidogyne javanica* (Mj) (J3; N1) was used in both gels as the control.

- Use 720 ml solution A + 180 ml solution B = 900 ml (4:1). Use 100 ml to prepare the esterase stain solution**.**

Stain solution

3.0 ml α-naphthyl acetate (1% in 50% acetone)
100 mg Fast Blue RR Salt

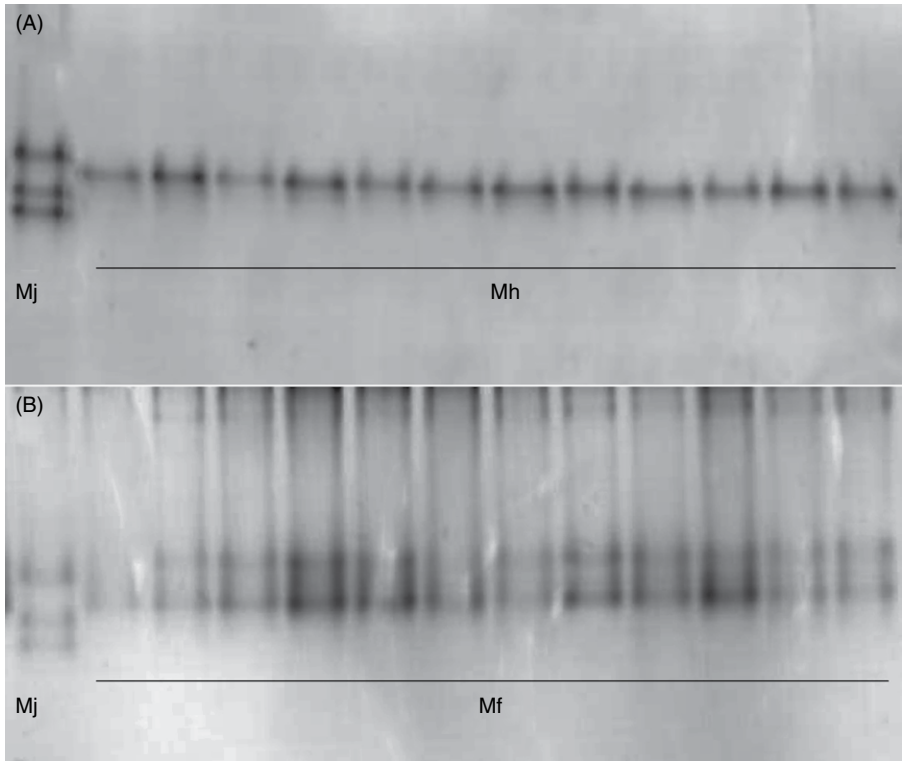

Fig. 11.8. Gels stained only for esterase detection. **(A)** *Meloidogyne hapla* (Mh) (H1). **(B)** *M. floridensis* (Mf) (F3). *Meloidogyne javanica* (Mj) (J3) was used in each gel as the control.

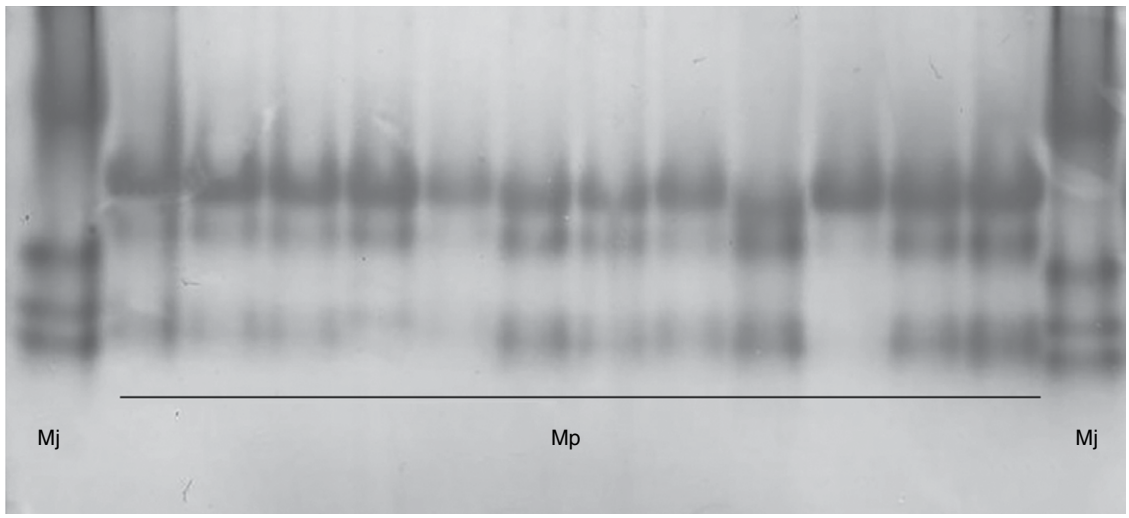

Fig. 11.9. Single gel stained for both esterase (EST) (upper bands) and malate dehydrogenase (MDH) (lower bands) detection showing the phenotypes found for *Meloidogyne partityla* (Mp) (Mp3; N1a). *Meloidogyne javanica* (Mj) (J3; N1) was used in each gel as the control.

- 100 ml 0.05 M potassium phosphate buffer, pH 6.0. This is enough for two gels. Mix the solution using a stirrer for 5 min and filter it (No. 2 Whatman) over vacuum. Add the 3.0 ml α-naphthyl acetate dropwise to the solution while stirring.

11.3.6.2 Staining solution for malate dehydrogenase

0.05 M TRIS-HCl, pH 8.6 (**stock solution**)
6.055 g TRIS Base
600 ml dH_2O
- Adjust pH to **8.6** with 6 N HCl. Bring volume up to 1000 ml with dH_2O. Keep in the refrigerator for up to 3 months.

Stain solution
50 ml 0.05 M TRIS-HCl, pH 8.6
0.01 g thiazolyl blue tetrazolium blue (MTT)
0.013 g β-nicotinamida adenine dinucleotide (β-NAD)
0.038 g L(–) malic acid
0.003 g phenazine methosulfate (PMS)
- Mix together using a stirrer for 5 min. This is enough for one gel.

11.3.6.3 Staining solution for glutamate-oxaloacetate transaminase (Harris and Hopkinson, 1976)

37 mg α-ketoglutaric acid
133 mg L-aspartic acid
0.5 g PVP-40
50 mg EDTA
1.42 g sodium phosphate (dibasic)
100 mg Fast Blue BB salt
100 ml dH_2O

11.3.6.4 Staining solution for superoxide dismutase (Ravindranath and Fridovich, 1975 cited by Esbenshade and Triantaphyllou (1985b))

100 ml 0.05M TRIS-HCl, pH 8.2
7.5 mg Sodium EDTA
4.0 mg Riboflavin
10 mg nitro blue tetrazolium chloride (NBT) or MTT

11.3.6.5 Fixative solution (20% ethanol and 10% glycerin)

20 ml ethanol (approx. 100%)
10 ml glycerin
70 ml dH_2O
Total = 100 ml. This is enough for two gels. Fix gels for at least 30 min at room temperature.

11.4 References

Brito, J.A., Kaur, R., Cetintas, R., Stanley, J.D., Mendes, M.L., McAvoy, E.J., Powers, T.O. and Dickson, D.W. (2008) Identification and isozyme characterization of *Meloidogyne* spp. infecting horticultural and agronomic crops and weed plants in Florida. *Nematology* 10, 757–766. DOI: 10.1163/156854108785787253

Bakker, J., Schots, A., Bouwman-Smits, L. and Gommers, F.L. (1988). Species-specific and thermostable proteins form second-stage larvae *Globodera rostochiensis* and *G. pallida. Phytopathology* 78, 300–305.
Cetintas, R., Lima, R.D., Mendes, M.L., Brito, J.A. and Dickson, D.W. (2003) *Meloidogyne javanica* on peanut in Florida. *Journal of Nematology* 35, 433–436.
Dalmasso, A. and Bergé, J.B. (1978) Molecular polymorphism and phylogenetic relationship in some *Meloidogyne* spp. Application to the taxonomy of *Meloidogyne. Journal of Nematology* 10, 323–332.
Dickson, D.W., Huisingh, D. and Sasser, J.N. (1971) Dehydrogenases, acid and alkaline phosphatases, and esterases for chemotaxonomy of selected *Meloidogyne*, *Ditylenchus*, *Heterodera*, *Aphelenchus* spp. *Journal of Nematology* 3, 1–16.
Esbenshade, P.R. and Triantaphyllou, A.C. (1985a) Use of enzyme phenotypes for identification of *Meloidogyne* species. *Journal of Nematology* 17, 6–20.
Esbenshade, P.R. and Triantaphyllou, A.C. (1985b) Electrophoretic methods for the study root-knot nematode enzymes. In: Barker, K.R., Carter, C.C. and Sasser, R.N. (eds) *An Advanced Treatise on* Meloidogyne. *Vol. 2 Methodology*. University of North Caroline State Graphics, Raleigh, North Carolina, USA, pp. 115–123.
Esbenshade, P.R. and Triantaphyllou, A.C. (1990) Isoenzyme phenotypes for the identification of Meloidogyne species. *Journal of Nematology* 22, 10–15.
Fleming, C.C. and Marks, R.J. (1983) The identification of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* by isoelectric focusing of protein on polyacrylamide gels. *Annals of Applied Biology* 103, 227–281. DOI: 10.1111/j.1744-7348.1983.tb02765.x
Fox, P.C. and Atkinson, H.A. (1984) Isoelectric focusing of general protein and specific enzymes from pathotypes of *Globodera rostochiensis* and *G. pallida*. *Parasitology* 88, 131–139.
Harris, H. and Hopkinson, D.A. (1976) *Handbook of Enzyme Electrophoresis in Human Genetics*. North-Holland Publishing Company, Amsterdam, The Netherlands.
Huettel, R.N., Dickson, D.W. and Kaplan, D.T. (1983) Biochemical identification of the two races of *Radopholus similis* by polyacrylamide gel electrophoresis. *Journal of Nematology* 15, 345–348.
Hussey, R.S. and Krusberg, L.R. (1971) Disc-electrophoresis patterns and soluble proteins of *Ditylenchus dipsaci* and *D. triformis*. *Journal of Nematology* 3, 79–84.
Hussey, R.S., Sasser, J.N. and Huisingh, D. (1972). Disc-electrophoretic studies of soluble proteins and enzymes of *Meloidogyne incognita* and *M. arenaria*. *Journal of Nematology* 4, 183–188.
Ibrahim, S.K. and Perry, R.N. (1993) Use of esterase patterns of females and galled roots for the identification of species of *Meloidogyne*. *Fundamental and Applied Nematology* 16, 187–191.
Ibrahim, S.K. and Rowe, J. (1995) Use of isoelectric focusing and polyacrylamide gel electrophoresis of nonspecific esterase phenotypes for the identification of cyst nematodes *Heterodera* species. *Fundamental and Applied Nematology* 18, 189–196.
Ibrahim, S.K., Perry, R.N. and Webb, R.M. (1995) Use of isoenzyme and protein phenotypes to discriminate between six *Pratylenchus* species from Great Britain. *Annals of Applied Biology* 126, 317–327. DOI: 10.1111/j.1744-7348.1995.tb05369.x
Ibrahim, S.K., Davies, K.G. and Perry, R.N. (1996) Identification of the root-knot nematode, *Meloidogyne incognita*, using monoclonal antibodies raised to non-specific esterases. *Physiological and Molecular Plant Pathology* 49, 79–88. DOI: 10.1006/pmpp.1996.0041
Karssen, G., Van Hoenselaar, T., Verkerk-Bakker, B. and Janssen, R. (1995) Species identification of cyst and root-knot nematodes from potato by electrophoresis of individual females. *Electrophoresis* 16, 105–109. DOI: 10.1002/elps.1150160119
Lawson, E.C., Carter, G.E. and Lewis Jr, S.A. (1984) Application of isoelectric focusing to the taxonomic identification of *Meloidogyne* spp. *Journal of Nematology* 16, 91–96.
Nobbs, J.M., Ibrahim, S.K. and Rowe, J. (1992) A morphological and biochemical comparison of the four cyst nematode species, *Heterodera elachista*, *H. oryzicola*, *H. oryzae* and *H. sacchari* (Nematoda: Heteroderidae) known to attack rice (*Oryza sativa*). *Fundamental and Applied Nematology* 15, 551–562.
Pozdol, R.F. and Noel, G.R. (1984) Comparative electrophoretic analyses of soluble proteins from *Heterodera glycines* races 1-4 and other *Heterodera* species. *Journal of Nematology* 16, 332–340.
Radice, A.D., Riggs, R.D. and Huang, F.H. (1988) Detection of intraspecific diversity of *Heterodera glycines* using isozymes phenotypes. *Journal of Nematology* 20, 29–39.
Sumiya, T., Hirata, K. and Yaegashi, T. (2002) Identification of three *Globodera* species in Japan by using isoelectric focusing method. *Research Bulletin of the Plant Protection Service, Japan* 38, 49–51.
Wharton, R.J., Storey, R.M.J. and Fox, P.C. (1983) The potential of some immunochemical and biochemical approaches to the taxonomy of potato cyst nematodes. In: Stone, A.J., Platt, H.M. and Khalil, L.F. (eds). *Concepts in Nematode Systematics*. Academic Press, London, UK, pp. 235–248.

12 Molecular Identification of Nematodes Using Polymerase Chain Reaction (PCR)

SERGEI A. SUBBOTIN

California Department of Food and Agriculture, Sacramento, California, USA; Center of Parasitology of A.N. Severtsov Institute of Ecology and Evolution, Moscow, Russia

12.1 Introduction

Molecular diagnostics are a vital component of the management of economically important pests, including plant-parasitic nematodes. Conventional methods for nematode identification rely on time-consuming morphological and morphometric analysis of several specimens of the target nematode. The accuracy and reliability of such identification depends largely on the experience and skill of the person making the diagnosis, and the number of such qualified and experienced nematode taxonomists is small and currently declining. Molecular methods of nematode identification provide accurate and alternative diagnostic approaches. Molecular diagnostics is a term used more specifically for the characterization of an organism based on information of its DNA or RNA structure.

Compared with biochemical approaches, molecular diagnostics has several advantages. It does not rely on expressed products and is not influenced by environmental conditions or development stage, and any stage (eggs, juveniles, females and males) can be used for diagnosis. It is much more sensitive than any biochemical technique and can be used with nanograms of DNA extracted from one nematode or even part of a nematode's body. It can also be used with various types of samples, such as soil extracts, plant material or formalin-fixed samples.

Various molecular techniques for diagnostics have been introduced to nematology during last decades, but the most popular is Polymerase Chain Reaction (PCR) based. PCR is a rapid, inexpensive and simple means of producing relatively large numbers of copies of DNA molecules via an enzyme catalyst. Once identified, target nematode DNA generated by PCR amplification can be characterized further by various analyses, including restriction fragment length polymorphism, when variation in sequences in PCR products can be revealed by restriction endonuclease digestion, or sequencing, which is a process of determining the order of

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
10.1079/9781786391759.0012

the nucleotide bases along a DNA strand. Identification of nematodes using PCR requires standard molecular biological equipment: a DNA thermal cycler, set of calibrated pipettes, vortex, centrifuge, horizontal gel electrophoresis box with power supply, freezer, microwave oven and UV light box with camera (Fig. 12.1).

The main DNA regions targeted for diagnostics of nematodes are nuclear ribosomal RNA genes. These include 18S, 28S and especially the Internal Transcribed Spacer 1 (ITS1) and Internal Transcribed Spacer 2 (ITS2), which are situated between 18S and 5.8S, and 5.8S and 28S rRNA genes, respectively. Genes of mitochondrial DNA (mtDNA), with their higher rate of mutations relative to rRNA genes, have great potential for identification of

Fig. 12.1. Laboratory equipment. **(A)** AirClean PCR workstation for preparation of PCR mixture. **(B, C)** Eppendorf PCR thermocycles. **(D)** Gel electrophoresis supplies and equipment. **(E)** Containers for EtBr staining. **(F)** Bio-Rad gel imaging system.

races and populations. Presently, partial cytochrome *c* oxidase subunit I (*COI*) gene of mtDNA is also widely used for nematode diagnostics. The *COI* gene is emerging as the standard barcode for many organisms. The bar-coding technique is based on the idea that a particular nucleotide sequence from a common gene can serve as a **unique identifier** for every species, and a single piece of DNA can identify all life forms on earth.

12.2 Preservation of Nematodes for Molecular Studies

The efficiency of DNA extraction from a sample depends on how the nematodes have been prepared and fixed for molecular analysis. Various fixation methods for molecular study have been proposed and described; however, the best approach is to use live nematodes for diagnostics. If the period between nematode extraction and molecular analysis is several days or weeks, nematodes may be kept at low temperatures (–20°C to +4°C) in a freezer or refrigerator before use. In some cases, quarantine regulations do not allow live nematodes to be kept and transported, so the nematodes should be killed gently using high temperature (75–80°C for 15 min) leaving the DNA undamaged. A sodium chloride (0.1 M NaCl) solution can be used at low or room temperature to store or send dead nematodes for several days. Often during long field sampling trips, it is not possible to keep nematodes at low temperatures and other methods should be used to save nematode DNA.

12.2.1 Dry preservation

Dry preservation is a simple method that has worked successfully with many nematode species, allowing DNA storage for many years. DNA is essentially stable when the sample is properly dried. Live nematodes are placed into a small Eppendorf tube with a drop of distilled water and the tube is kept open at room temperature or gently heated until the water has evaporated. Dead and dried nematodes will be at the bottom of the tube. The tube with nematodes could be stored at room temperature. Before starting DNA extraction, add a few drops of water to the tube and wait for a few minutes for the specimens to rehydrate.

12.2.2 Ethanol (ethyl alcohol) preservation

Ethanol (ethyl alcohol) mixed with distilled water is a good preserving agent. Nematodes preserved in different concentrations (75–95%) of ethanol and stored at ambient temperature can be successfully used in PCR and other molecular analyses. Nematodes fixed in ethanol should always be carefully washed in distilled water before DNA extraction.

12.2.3 DESS preservation

DESS is a solution containing dimethyl sulfoxide, disodium EDTA and saturated NaCl. DESS offers the advantage of preserving both the morphology and DNA with one solution, as opposed to previous sampling methodology that required collection of separate sub-samples in ethanol and formalin for integrative studies. Nematodes picked straight out of DESS can be used successfully for PCR after several months storage (maximum storage time is 7 months). Amplification of DNA fragments from 800 to 1800 bp was 80% successful and sequencing success from these amplicons was greater than 90% for all nematodes preserved in DESS solution between 3 days and 7 months (Yoder *et al.*, 2006). Specimens fixed in DESS solution should be washed in distilled water before DNA extraction.

12.2.3.1 Protocol for preparing DESS solution

After http://www.faculty.ucr.edu/~pdeley/lab/melissa/DESS_protocol_f.doc (See also Eisenback and Hunt, Chapter 5, this volume.)

- For a 250 ml solution of DESS, measure out 23.265 g of disodium EDTA with FW 372.24. (This may vary depending on the FW of your EDTA salt.) Add 50 ml of deionized water to the EDTA salt and stir. Make sure disodium EDTA salt is used, otherwise more NaOH is needed to pH the EDTA.

- Make 1 M NaOH to pH the EDTA. The EDTA should be around pH 3.0 or 4.0 to begin with. It will take approximately 50 ml of 1 M NaOH to bring the EDTA to pH 8.0. The EDTA will then begin to dissolve slowly. Heat to 30°C.
- Once all the EDTA salt is dissolved, bring the volume up to 200 ml with deionized water. Then add the 20% DMSO by volume, which is 50 ml for a 250 ml solution. Return to a beaker and stir for a few minutes.
- Add NaCl until the solution is saturated (i.e. it no longer dissolves); heating will help dissolve the salt. Pour the solution into a bottle leaving most of the salt crystals in the beaker.
- To extract the samples from DESS, pick the nematodes out of the solution and place them in a small Petri dish with distilled water for a few minutes to remove any salt or DESS that might be attached. If the DESS is not completely washed off, the dimethyl sulfoxide and EDTA will inhibit the PCR reaction.

12.3 DNA Extraction

The critical step in molecular identification procedures is the preparation of the template DNA from a nematode sample. Successful molecular identification requires the availability of genomic material of an appropriate quality and concentration. The aim of this procedure is to expose the DNA molecules for further analyses and remove materials that may inhibit subsequent reactions.

Several protocols for the extraction of nucleic acids from nematodes are available (e.g. Curran *et al.*, 1985; Caswell-Chen *et al.*, 1992; Blok *et al.*, 1997). Choosing an appropriate DNA extraction method usually depends on the amount of available nematode material and the method used. Several general conditions should be considered for selection of DNA extraction method: (i) avoid losing DNA and retain as much DNA as possible; (ii) final DNA should be free from inhibiting materials; (iii) the method should be simple with minimal steps and tube changes to avoid laboratory contamination; (iv) the method should involve only limited exposure to toxic chemicals; and (v) the method should be relatively inexpensive and non-labour intensive.

DNA extraction generally follows three basic steps: (i) disruption of nematode cuticle, cell walls and membranes; (ii) separation the DNA from other cell components; and (iii) isolation of the DNA. These three steps or some variation of them can be found in all DNA extraction methods.

Using different extraction methods and commercial kits, nematode DNA can be obtained directly from soil samples (Waite *et al.*, 2003; Yan *et al.*, 2008, 2013; Goto *et al.*, 2009; Sato *et al.*, 2010; Baidoo *et al.*, 2017). Furthermore, extraction of DNA from formalin-fixed material or nematodes embedded in glycerin on slides provides a new opportunity for molecular examination of reference materials (Thomas *et al.*, 1997; Bhadury *et al.*, 2005; Rubtsova *et al.*, 2005).

12.3.1 Protocols for DNA extraction from nematodes

Protocol 1: DNA extraction using proteinase K with Worm Lysis Buffer (WLB) (Waeyenberge *et al.*, 2000)

- Pick a single or several nematodes and place in a 10 µl drop of double distilled water on a glass slide under a dissecting microscope.
- Cut nematodes into three or four pieces with a needle or scalpel.
- Transfer worm pieces with water to a sterile 0.2 ml Eppendorf tube containing 8 µl of WLB (50 mM KCl, 10 mM Tris, pH 8.2, 2.5 mM $MgCl_2$, 0.45% NP40 (Fisher Scientific), 0.45% Tween 20 (Merck) and 0.01% gelatine) and 2 µl of proteinase K (600 µg ml^{-1}).
- Freeze at –80°C for 10 min.
- Incubate at 65°C for 1 h and then heat at 95°C for 15 min.
- Centrifuge for 1 min at maximum speed to remove debris.
- Use 1–4 µl of the supernatant for PCR.

Protocol 2: DNA extraction using proteinase K with 10× PCR buffer (Subbotin *et al.*, 2018)

This is modification of the method of Waeyenberge *et al.* (2000), where WLB is replaced by 10x PCR buffer without losing DNA extraction efficiency.

- Pick a single or several nematodes and place in a 20 µl drop of double distilled water on a glass slide under a dissecting microscope.
- Cut nematodes into three or four pieces with a needle or scalpel. Nematodes in water can also be crushed under a cover slip with careful pressure on the slip.
- Transfer worm pieces with water to a sterile 0.2 ml Eppendorf tube and add 2 µl of 10× PCR buffer and 3 µl of proteinase K (600 µg ml^{-1}).
- Incubate at 65°C for 1 h and then heat at 95°C for 15 min.
- Centrifuge for 1 min at maximum speed to remove debris.
- Use 1–4 µl of the supernatant for PCR.

Protocol 3: DNA extraction using NaOH (Floyd *et al.*, 2002)

- Pick individual nematodes directly into 20 µl of 0.25 M NaOH in a 0.2 ml Eppendorf tube and keep at room temperature from several minutes to several hours.
- Heat the lysate for 3 min at 95°C.
- Add 4 µl of HCl and 10 µl of 0.5 M Tris-HCl buffered at pH 8.0 to neutralize the base.
- Add 5 µl of 2% Triton X-100.
- Heat the lysate for 3 min at 95°C.
- Use 0.5–2.0 µl of lysate for PCR.

Protocol 4: DNA extracted from archived nematodes using an extended hot lysis protocol with Qiagen DNeasy Tissue Kit (Chase *et al.*, 1998; Bhadury *et al.*, 2007)

- Carefully take nematodes off the microscope slides with a sterilized scalpel and place into 0.5 ml PCR tubes containing 200 µl of animal tissue lysis buffer (also known as ATL) from the Qiagen DNeasy Tissue Kit.
- Incubate tubes at 56°C for 24 h.
- Add 5 µl of proteinase K (50 mg ml^{-1}) and an additional 80 µl of the ATL buffer to each tube and incubate for another 72 h at 55°C.
- Complete the extraction procedure according to the DNeasy kit following the manufacturer's instructions. Finally, elute the DNA in 80 µl of MilliQ water.
- Immediately store templates at –20°C until further use.
- Use 5 µl aliquots of the extracted DNA for the PCR.

Effective DNA extraction can be achieved by using commercial kits developed by Qiagen, Promega and other companies.

12.4 PCR Technique

The PCR technique has become one of the most widely used techniques for studying the genetic diversity of nematodes and their identification. PCR is a rapid, inexpensive and simple means of producing large numbers of copies of DNA molecules. Any DNA fragment can be amplified and detected by PCR. The PCR method requires a DNA template (starting material) containing the region to be amplified, two oligonucleotide primers flanking this target region, DNA polymerase and four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) mixed in a buffer containing magnesium ions ($MgCl_2$).

A primer is a short oligonucleotide, containing usually about two dozen nucleotides, that is complementary to the 3′ end of each strand of the fragment that should be amplified. Primers anneal to the denatured DNA template and provide an initiation site for the elongation of the new DNA molecule. Universal primers are those complementary to a particular set of DNA for a wide range of organisms (Table 12.1); primers matching only to certain species are called species-specific primers (Table 12.2). When sequences of the flanking regions of the amplified fragment are unknown, PCR with degenerate primers (i.e. primers containing a number of options at several positions in the sequence that allows annealing and amplification of a variety of related sequences) can be applied. The universal primers for rRNA genes that are currently used for diagnostics of plant-parasitic nematodes are given in Table 12.1. The primers for amplification of some mtDNA genes are given in Table 14.1 (Humphreys-Pereira *et al.*, Chapter 14, this volume).

PCR is performed in a tube in a thermocycler with programmed heating and cooling. The procedure consists of a succession of three steps determined by temperature conditions: template denaturation (95ºC for 3–4 min), primer annealing (55–60ºC for 30 s to 2 min), and extension of the DNA chain (72ºC for 30 s to 2 min). PCR is carried out for 30–40 cycles. As the result of PCR, a single target molecule of DNA is amplified into more than a billion copies. The amplified products are electrophoretically separated according to their size on agarose or polyacrylamide gels and visualized using ethidium bromide (EtBr) or other DNA-staining dyes, which interact with double-stranded DNA and causes it to fluoresce under UV radiation. Once identified, the target nematode DNA generated by PCR amplification can be characterized further by various analyses.

Table 12.1. Some universal primer combinations used for amplification of ribosomal RNA genes of nematodes.

Primer combination and code (direction)[a]	Primer sequence (5′-3′)	Amplified region	References
G18SU (f)	GCT TGC CTC AAA GAT TAA GCC	18S rRNA	Blaxter *et al.* (1998)
R18Tyl1 (r)	GGT CCA AGA ATT TCA CCT CTC		Chizhov *et al.* (2006)
F18Tyl2 (f)	CAG CCG CGG TAA TTC CAG C	18S rRNA	Chizhov *et al.* (2006)
R18Tyl2 (r)	CGG TGT GTA CAA AGG GCA GG		
988F (f)	CTC AAA GAT TAA GCC ATG C	18S rRNA	Holterman *et al.* (2006)
1912R (r)	TTT ACG GTC AGA ACT AGG G		
1096F (f)	GGT AAT TCT GGA GCT AAT AC	18S rRNA	Holterman *et al.* (2006)
1912R (r)	TTT ACG GTC AGA ACT AGG G		
1813F (f)	CTG CGT GAG AGG TGA AAT	18S rRNA	Holterman *et al.* (2006)
2646R (r)	GCT ACC TTG TTA CGA CTT TT		
SSU_F_04	GCT TGT CTC AAA GAT TAA GCC	18S rRNA	Blaxter *et al.* (1998)
SSU_R_09	AGC TGG AAT TAC CGC GGC TG		
SSU_F_22	TCC AAG GAA GGC AGC AGG C	18S rRNA	Blaxter *et al.* (1998)
SSU_R_13,	GGG CAT CAC AGA CCT GTT A		
SSU_F_23	ATT CCG ATA ACG AGC GAG A	18S rRNA	Blaxter *et al.* (1998)
SSU_R_81	TGA TCC WKC YGC AGG TTC AC		
designated Nem_18S_F	CGC GAA TRG CTC ATT ACA ACA GC	18S rRNA	Floyd *et al*. (2005)
Nem_18S_R	GGG CGG TAT CTG ATC GCC		
18S-CL-F3	CTT GTC TCA AAG ATT AAG CCA TGC AT	18S rRNA + ITS1-5.8S-ITS2 rRNA + 28S rRNA	Carta and Li (2018, 2019)
28S-CL-R	CAG CTA CTA GAT GGT TCG ATT AGT C		
18S (f)	TTG ATT ACG TCC CTG CCC TTT	ITS1-rRNA	Vrain *et al.* (1992)
rDNA1.58S (r)	ACG AGC CGA GTG ATC CAC CG		Szalanski *et al.* (1997)
TW81 (f)	GTT TCC GTA GGT GAA CCT GC	ITS1-rRNA	Curran *et al.* (1994)
5.8SM5 (r)	GGC GCA ATG TGC ATT CGA		Zheng *et al.* (2000)
18S (f)	TTG ATT ACG TCC CTG CCC TTT	ITS1-5.8S-ITS2 rRNA	Vrain *et al.* (1992)
26S (r)	TTT CAC TCG CCG TTA CTA AGG		
F194 (f)	CGT AAC AAG GTA GCT GTA G	ITS1-5.8S-ITS2 rRNA	Ferris *et al.* (1993)
F195 (r)	TCC TCC GCT AAA TGA TAT G		
TW81 (f)	GTT TCC GTA GGT GAA CCT GC	ITS1-5.8S-ITS2 rRNA	Curran *et al.* (1994)
AB21 (r)	ATA TGC TTA AGT TCA GCG GGT		
D2A (f)	ACA AGT ACC GTG AGG GAA AGT TG	D2-D3 of 28S rRNA	Nunn (1992)
D3B (r)	TCG GAA GGA ACC AGC TAC TA		
D2Tyl (f)	GAG AGA GTT AAA NAG BAC GTG A	D2-D3 of 28S rRNA	Chizhov *et al.* (2012)
D3B (r)	TCG GAA GGA ACC AGC TAC TA		Nunn (1992)
D2A (f)	ACA AGT ACC GTG AGG GAA AGT TG	D2 of 28S rRNA	Nunn (1992)
D2B (r)	GAC CCG TCT TGA AAC ACG GA		

[a]f, forward; r, reverse.

Table 12.2. Species-specific primers for conventional PCR used for diagnostics of some plant-parasitic nematodes.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Aphelenchoides besseyi	AbF5 – ATG TGT AAG TAG AGC GTT ATA AbR5 – ATT CGC CGT TTT TAA GGC G-	18S rRNA	~ 340 bp	Devran *et al.* (2017)
Aphelenchoides fragariae	AFragF1 – GCA AGT GCT ATG CGA TCT TCT AfragR1 – GCC ACA TCG GGT CAT TAT TT	ITS rRNA	~ 169 bp	McCuiston *et al.* (2007)
Aphelenchoides ritzemabosi	BSF – TCG ATG AAG AAC GCA GTG AAT T ArtR – CTC CAC ACG CCG ACC GA	ITS rRNA	~ 208 bp	Cui *et al.* (2010)
Bursaphelenchus cocophilus	BC1F – AAC TAC CGT CTT CCG CTG TCG BC1R - TTG AGC ACC AAC ACG CCG TCA	ITS rRNA	~ 528 bp	Silva *et al.* (2016)
Bursaphelenchus fraudulentus	FF – GTG ATG GGT TTG CGG GCG GCG FR – CAA CCA ATG CAC ACC AAC CAA	ITS rRNA	~ 617 bp	Filipiak *et al.* (2010)
Bursaphelenchus mucronatus	MF - TCCGGCCATATCTCTACGAC MR - GTTTCAACCAATTCCGAACC	ITS rRNA	~ 210 bp	Matsunaga and Togashi (2004)
Bursaphelenchus xylophilus	XF - ACGATGATGCGATTGGTGAC XR - TATTGGTCGCGGAACAAACC	ITS rRNA	~ 557 bp	Matsunaga and Togashi (2004)
Bursaphelenchus mucronatus	Y01F - AGT CCG TGC CTT TGC TCT AGC Y01R - CCG AAG TGT CTC CAG CGA AAT	SCAR	~ 609 bp	Chen *et al.* (2011)
Bursaphelenchus xylophilus	BZ2 – TCA CGA TGA TGC GAT TGG TG BF3 – AGA AGA TCT TGG TCG CGG AA	ITS rRNA	~ 580 bp	Jiang *et al.* (2005)
Ditylenchus destructor	D2 – TGG ATC ACT CGG CGG CTC GTA GA D1 – ACT GCT CTG CGT TTG GCT TCA	D2-D3 of 28S rRNA	~ 346 bp	Liu *et al.* (2007)
Ditylenchus dipsaci	DitNF1 – TTA TGA CAA ATT CAT GGC GG rDNA2 - TTT CAC TCG CCG TTA CTA AGG	ITS rRNA	~ 263 bp	Subbotin *et al.* (2005)
Ditylenchus dipsaci	U831 - AAY AAR ACM AAG CCN TYT GGA C Dipsaci-hsp90R - GWG TTA WAT AAC TTG GTC RGC	Hsp90	~ 182 bp	Madani *et al.* (2015)
Ditylenchus dipsaci	H05 - TCA AGG TAA TCT TTT TCC CCA CT H06 - CAACTG CTA ATG CGT GCT CT	SCAR	~ 242 bp	Esquibet *et al.* (2003)
Ditylenchus dipsaci	DdpS1 - TGG CTG CGT TGA AGA GAA CT rDNA2 - TTT CAC TCG CCG TTA CTA AGG	ITS rRNA	~ 517 bp	Kerkoud *et al.* (2007)
Ditylenchus dipsaci	DITuniF – CTG TAG GTG AAC CTG C DITdipR – GAC ATC ACC AGT GAG CAT CG	ITS rRNA	~ 148 bp	Jeszke *et al.* (2015)
Ditylenchus gigas	D09 - CAA AGT GTT TGA TCG ACT GGA D10 - CAT CCC AAA ACA AAG AAA GG	SCAR	~ 198 bp	Esquibet *et al.* (2003)

(*Continued*)

Table 12.2. Continued.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Ditylenchus gigas	DITuniF – CTG TAG GTG AAC CTG C DITgigR – GAC CAC CTG TCG ATT C	ITS rRNA	~ 270 bp	Jeszke *et al.* (2015)
Globodera rostochiensis	PITSr3 – AGC GCA GAC ATG CCG CAA ITS5 – GGA AGT AAA AGT CGT AAC AAG G	ITS rRNA	~ 434 bp	Bulman and Marshall (1997)
Globodera rostochiensis	GGT GAC TCG ACG ATT GCT GT GCA GTT GGC TAG CGA TCT TC	ITS rRNA	~ 391 bp	Mulholland *et al.* (1996)
Globodera pallida	PITSp4 – ACA ACA GCA ATC GTC GAG ITS5 – GGA AGT AAA AGT CGT AAC AAG G	ITS rRNA	~ 265 bp	Bulman and Marshall (1997)
Globodera pallida	GGT GAC TCG ACG ATT GCT GT GCA GTT GGC TAG CGA TCT TC	ITS rRNA	~ 238 bp	Mulholland *et al.* (1996)
Heterodera avenae	AVEN-COIF - GGG TTT TCG GTT ATT TGG AVEN-COIR - CGC CTA TCT AAA TCT ATA CCA	COI	~ 109 bp	Toumi *et al.* (2013a)
Heterodera filipjevi	FILI-COIF - GTA GGA ATA GAT TTA GAT AGT C FILI-COIR - TGA GCA ACA ACA TAA TAA G	COI	~ 245 bp	Toumi *et al.* (2013a)
Heterodera filipjevi	HfF1 – CAG GAC GAA ACT CAT TCA ACC AA HfR1 – AGG GCG AAC AGG AGA AGA TTA GA	SCAR	~ 646 bp	Peng *et al.* (2013)
Heterodera latipons	Hlat-actF - ATG CCA TCA TTA TTC CTT Hlat-actR - ACA GAG AGT CAA ATT GTG	actin	~ 204 bp	Toumi *et al.* (2013b)
Heterodera glycines	JBG1 – TGG TTT AGT TAG ATT AAC TAT C JB3R – TCC AAA CTW GCG TTA CTY AG	COI	~ 339 bp	Ko *et al.* (2017)
Heterodera glycines	SCNFI – GGA CCC TGA CCA AAA AGT TTC CGC SCNRI – GGA CCC TGA CGA GTT ATG GGC CCG	SCAR	~ 477 bp	Ou *et al.* (2008)
Heterodera glycines	GlyF1 – TTA CGG ACC GTA ACT CAA 26S – TTT CAC TCG CCG TTA CTA AGG	ITS rRNA	~ 181 bp	Subbotin *et al.* (2001)
Heterodera schachtii	JBS1 – GGA TAA TTT ATG CTA TTA TC JB3R –TCC AAA CTW GCG TTA CTY AG	COI	~ 339 bp	Ko *et al.* (2017)
Heterodera schachtii	SHF6 – GTT CTT ACG TTA CTT CCA AB28 - ATA TGC TTA AGT TCA GCG GGT	ITS rRNA	~ 200 bp	Amiri *et al.* (2002)
Hoplolaimus columbus	Hoc-1f – AAC CTG CTG CTG GAT CAT TA HC-1r – TCA GCA CAC AAT GGT ACC TTT	ITS1 rRNA	~ 580 bp	Bae *et al.* (2009)
Hoplolaimus galeatus	Hoc-1f – AAC CTG CTG CTG GAT CAT TA HG-2r – TCC TCG TTC ACA CAT TGA CA	ITS1 rRNA	~ 120 bp	Bae *et al.* (2009)

(*Continued*)

Table 12.2. Continued.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Hoplolaimus magnistylus	Hoc-1f – AAC CTG CTG CTG GAT CAT TA HM-3r – AGA CTG GAC GGC CAA AGT T	ITS1 rRNA	~ 340 bp	Bae *et al.* (2009)
Longidorus attenuatus	GenF - TTG ATT ACG TCC CTG CCC TTT GT Latten3 - TTC CCT TTT CCC TGA TTA TAA TTT TCT ATC	ITS1 rRNA	~ 419 bp	Hübschen *et al.* (2004)
Longidorus elongatus	GenF - TTG ATT ACG TCC CTG CCC TTT GT Lelong1 - TTA TCG TAC GTA TTC CCA GTT CT	ITS1 rRNA	~ 847 bp	Hübschen *et al.* (2004)
Longidorus macrosoma	GenF - TTG ATT ACG TCC CTG CCC TTT GT Lmacro2 - GTT CCC GAC GAT TAT TTT TGT	ITS1 rRNA	~ 705 bp	Hübschen *et al.* (2004)
Longidorus helveticus	GenF - TTG ATT ACG TCC CTG CCC TTT GT Lhel1 - CCG CAT CTC TTT ATT TCC GAC CAT CAA CC	ITS1 rRNA	~ 369 bp	Hübschen *et al.* (2004)
Longidorus profundorum	GenF - TTG ATT ACG TCC CTG CCC TTT GT Lprof2 - TTA TTA TTT TTC AGG CTC TAC CTT TCG C	ITS1 rRNA	~ 1071bp	Hübschen *et al.* (2004)
Longidorus sturhani	GenF - TTG ATT ACG TCC CTG CCC TTT GT Lstur - TTT TCC CCA CTA ATA CTC CCT CGT T	ITS1 rRNA	~ 667 bp	Hübschen *et al.* (2004)
Meloidogyne arenaria	Far – TCG GCG ATA GAG GTA AAT GAC Rar – TCG GCG ATA GAC ACT ACA AACT	SCAR	~ 420 bp	Zijlstra *et al.* (2000)
Meloidogyne chitwoodi	Fc - TGG AGA GCA GCA GGA GAA AGA- Rc - GGT CTG AGT GAG GAC AAG AGT A	SCAR	~ 800 bp	Zijlstra (2000)
Meloidogyne enterolobii	Me-F - AACTTTTGTGAAAGTGCCGCTG Me-R - TCAGTTCAGGCAGGATCAACC	IGS rRNA	~ 200 bp	Long *et al.* (2006)
Meloidogyne exigua	Ex-D15-F – CAT CCG TGC TGT AGC TGC GAG Ex-D15-R – CTC CGT GGG AAG AAA GAC TG	SCAR	562 bp	Randig *et al.* (2002)
Meloidogyne fallax	Ff - CCA AAC TAT CGT AAT GCA TTA TT Rf -GGA CAC AGT AAT TCA TGA GCT AG	SCAR	~ 515 bp	Zijlstra *et al.* (2000)
Meloidogyne hapla	Fh – TGA CGG CGG TGA GTG CGA Rh – TGA CGG CGG TAC CTC ATA G	SCAR	610 bp	Zijlstra (2000)
Meloidogyne incognita	Finc – CTC TGC CCA ATG AGC TGT CC Rinc – CTC TGC CCT CAC ATT AGG	SCAR	~ 1200 bp	Zijlstra *et al.* (2000)

(*Continued*)

Table 12.2. Continued.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Meloidogyne incognita	MI-F – GTG AGG ATT CAG CTC CCC AG MI-R – ACG AGG AAC ATA CTT CTC CGT CC	SCAR	~ 955 bp	Meng *et al.* (2004)
Meloidogyne incognita	F – TAG GCA GTA GGT TGT CGG G R - CAG ATA TCT CTG CAT TGG TGC	SCAR	~ 1350 bp	Dong *et al.* (2001)
Meloidogyne incognita	Inc-K14-F – GGG ATG TGT AAA TGC TCC TG Inc-K14-R – CCC GCT ACA CCC TCA ACT TC	SCAR	~ 399 bp	Randig *et al.* (2002)
Meloidogyne javanica	Fjav – GGT GCG CGA TTG AAC TGA GC Rjav – CAG GCC CTT CAG TGG AAC TAT AC	SCAR	~ 620 bp	Zijlstra *et al.* (2000)
Meloidogyne naasi	N-ITS – CTC TTT ATG GAG AAT AAT CGTR195 – CCT CCG CTT ACT GAT ATG	ITS rRNA	433 bp	Zijlstra *et al.* (2004)
Nacobbus spp.	NacF - GAT CAT TAC ACG TAC CGT GAT GGT C NacR - CTG CTC AAC CAC GCA TAG ACG	ITS rRNA	141-173 bp	Atkins *et al.* (2005)
Paralongidorus maximus	GenF - TTG ATT ACG TCC CTG CCC TTT GT Pmax1 - TGC ATT TCA CCA CTT CTC ACT C	ITS1 rRNA	~ 649 bp	Hübschen *et al.* (2004)
Paratrichodorus allius	BL18 – CCC GTC GMT ACT ACC GAT T PAR2 - CCG TYC AAA CGC GTA TAT GAT C	ITS rRNA	~ 432 bp	Riga *et al.* (2007)
Paratrichodorus teres	BL18 – CCC GTC GMT ACT ACC GAT T PTR4 – CCT GAC AAG CTT GCA CTAG C	ITS rRNA	~ 677 bp	Riga *et al.* (2007)
Pratylenchus brachyurus	18S - TTG ATT ACG TCC CTG CCC TTT ACM7R – GCW CCA TCC AAA CAA YGA G	ITS1 rRNA	~ 267 bp	Machado *et al.* (2007)
Pratylenchus bolivianus	TW81 - GTT TCC GTA GGT GAA CCT GC P-bolivR1 -ATA GCG CAC TGG CGC AGC ATA	ITS rRNA	~ 295 bp	Troccoli *et al.* (2016)
Pratylenchus crenatus	PCR22 (f) – AAA GCC TGA ATG CCC TGA G PCR22 (r) – AAA TTG AAA GAG GTC GGT CGT	ITS rRNA	~ 610 bp	Mekete *et al.* (2011)
Pratylenchus jaehni	Pj1F – TGG TCA ATG AAT GTT ACG 5818 – ACG ARC CGA GTG ATC CAC	ITS1 rRNA	~ 476 bp	Consoli *et al.* (2012)
Pratylenchus neglectus	PNEG – ATG AAA GTG AAC ATG TCC TC D3B -TCG GAA GGA ACC AGC TAC TA	D3 of 28S rRNA	~ 290 bp	Al-Banna *et al.* (2004)
Pratylenchus neglectus	PNEG-F1 – CGC AAT GAA AGT GAA CAA TGT C D3B5- AGT TCA CCA TCT TTC GGG TC	D3 of 28S rRNA	~ 144 bp	Yan *et al.* (2008)

(*Continued*)

Table 12.2. Continued.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Pratylenchus oleae	Poleae-fw1 – GAC AGA TTA GAA TGG AAT CTG TTC G Poleae-rv1 – ATC GCT TTT GGA TTC AAT AAT ATA	ITS rRNA	~ 547 bp	Palomares-Rius *et al.* (2014)
Pratylenchus parazeae	PpzF – CTG CTG CTG GAT CAT TAC ATT PpzR –TCA AAT AGA CAT GCC CCA AT	ITS rRNA	~ 570 bp	Wang *et al.* (2015)
Pratylenchus penetrans	PPEN – TAA AGA ATC CGC AAG GAT AC D3B -TCG GAA GGA ACC AGC TAC TA	D3 of 28S rRNA	~ 278 bp	Al-Banna *et al.* (2004)
Pratylenchus penetrans	PP5 (f) –ACA TGG TCG ACA CGG TGA TA PP5 (r) - TGT TGC GCA AAT CCT GTT TA	beta-1,4-endoglu-canase	~ 520 bp	Mekete *et al.* (2011)
Pratylenchus penetrans	PpenA – TGA CTA TAT GAC ACA TTT RAA CTT G AB28 -ATA TGC TTA AGT TCA GCG GGT	ITS rRNA	~ 660 bp	Waeyenberge *et al.* (2009)
Pratylenchus penetrans	PP1 – ATG ATG GAA GTG TCC GCC T PP2 – CCC AAC GAC GGT CAA AAG G	ITS rRNA	~ 462 bp	Uehara *et al.* (1998)
Pratylenchus scribneri	PSCR – AAA GTG AAC GTT TCC ATT TC D3B -TCG GAA GGA ACC AGC TAC TA	D3 of 28S rRNA	~ 286 bp	Al-Banna *et al.* (2004)
Pratylenchus scribneri	PsF7 – AGT GTT GCT ATA ATT CAT GTA AAG TTG C PsR7 –TGG CCA GAT GCG ATT CGA GAG GTG T	ITS rRNA	~ 136 bp	Huang and Yan (2017)
Pratylenchus speijeri	TW81 - GTT TCC GTA GGT GAA CCT GC speijeri–specific – GTG CAC TGA TGT TAT TAT GTA TGG	ITS rRNA	~ 102 bp	De Luca *et al.* (2012)
Pratylenchus thornei	PTHO – GAA AGT GAA GGT ATC CCT CG D3B -TCG GAA GGA ACC AGC TAC TA	D3 of 28S rRNA	~ 288 bp	Al-Banna *et al.* (2004)
Pratylenchus thornei	Pthf - TTC GGA AGA CAA TAA ATC Pthr - TCC AAA ATG AAA TAA TAA A	SCAR	~ 1078 bp	Carrasco-Ballesteros *et al.* (2007)
Pratylenchus vulnus	PVUL – GAA AGT GAA CGC ATC CGC AA D3B -TCG GAA GGA ACC AGC TAC TA	D3 of 28S rRNA	~ 287 bp	Al-Banna *et al.* (2004)
Pratylenchus zeae	TW81 - GTT TCC GTA GGT GAA CCT GC P-zeaeR1 - TAC GCA TAC RGT TCT GCT CAT	ITS rRNA	~ 560 bp	Troccoli *et al.* (2016)
Radopholus similis	PF – CTA CAA ATG TGA CGC GAA PR – CAA TCT GCA CAA TGA ACA TAC	ITS rRNA	~ 500 bp	Liu *et al.* (2011)
Radopholus similis	RsimF – GAT TCC GTC CTT TGG TGG GCA RsimR – GAA CCA GGC GTG CCA GAG G	ITS rRNA	~ 398 bp	Ravindran *et al.* (2011)

(*Continued*)

Table 12.2. Continued.

Nematode species	Primer code and sequence (5′-3′)	Gene fragment	Amplified size	References
Rotylenchulus reniformis	Ren240F – ACC GGC TTA ATT GCA ATG GT Ren240R – ACA ACT GCT CAA CAA CGC AG	ITS rRNA	~ 240 bp	Sayler *et al.* (2012)
Rotylenchulus reniformis	D2A - ACA AGT ACC GTG AGG GAA AGT TG Rrenif-R1A - GAA AAG GCC TAC CCA ATG TG	D2-D3 of 28S rRNA	~ 142 bp	Van den Berg *et al.* (2016)
Rotylenchus robustus	TW81 - GTT TCC GTA GGT GAA CCT GC R-robustus - GACGTGGACATCATACAGTC	ITS rRNA	~ 438 bp	Cantalapiedra-Navarrete *et al.* (2013)
Scutellonema bradys	TW81 - GTT TCC GTA GGT GAA CCT GC S-bradys – GTG ATG GCT AAA CCA CAT TC	ITS rRNA	~ 250 bp	Van den Berg *et al.* (2013)
Scutellonema brachyurus	TW81 - GTT TCC GTA GGT GAA CCT GC S-brachyurus-type A – GCT GAA GTG ACA GCC CAA CTT	ITS rRNA	~ 185 bp	Van den Berg *et al.* (2013)
Tylenchulus semipenetrans	TW81 - GTT TCC GTA GGT GAA CCT GC Semipenetrans – GGA CTC TGC TCA ACC TGG TAG A	ITS rRNA	~ 113 bp	Tanha Maafi *et al.* (2012)
Xiphinema diversicaudatum	TW81 - GTT TCC GTA GGT GAA CCT GC Xip-diver-ITS - GAA TAA ACA CCT TTC AAC GCT C	ITS rRNA	~ 864 bp	Chizhov *et al.* (2014)
Xiphinema index	I27 – GAG TCG TAA CGT TTC TCG TCT ATC AGG A-ITS1 – GAA TAG CCA CCT AGT GAG CCG AGC A	ITS rRNA	~ 340 bp	Wang *et al.* (2003)
Xiphinema vuittenezi	V18 – GTG GAA CGA AAA GAC CTC A-ITS1 – GAA TAG CCA CCT AGT GAG CCG AGC A	ITS rRNA	~ 591 bp	Wang *et al.* (2003)
Xiphinema italiae	ITA26 – GAA ATA AGA ACC CTG AAA AAG ATA GG A-ITS1 – GAA TAG CCA CCT AGT GAG CCG AGC A	ITS rRNA	~ 414 bp	Wang *et al.* (2003)

PCR with specific primers enables the detection of species in a nematode mixture by a single PCR test. Oligonucleotide primers for this PCR are designed to bind to regions of the gene that are conserved over the particular taxon or a group of taxa, so that it may be species-specific or it may target a group of species that differ from other such groups. Detection of a specific size amplicon in a gel indicates the presence of a certain species within a sample (Fig. 12.2). This PCR type constitutes a major development in DNA diagnostics. It enables the detection not only of a single species but also of several species in a nematode mixture by a single PCR test (multiplex PCR), provided several sets of specific primers for different species are mixed.

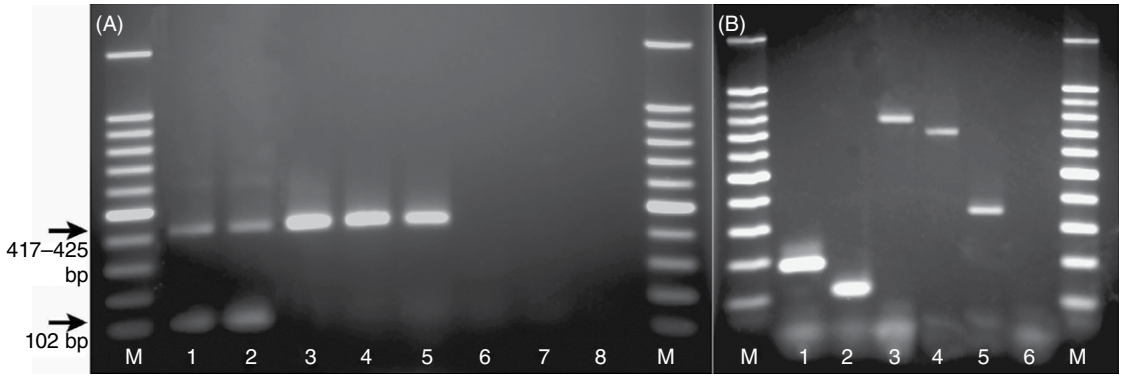

Fig. 12.2. **(A)** Duplex PCR with the *Pratylenchus coffeae* group-specific and *P. speijeri* species-specific primers. Lanes: M, 100 bp DNA marker (Promega); 1–4, *Pratylenchus* sp. C1; 5, *P. coffeae*; 6, *P. neglectus*; 7, *P. brachyurus*; 8, control without DNA. Arrows indicate a group-specific band for *P. coffeae* species complex (417–425 bp) and a species-specific band for *P. speijeri* (102 bp). (After De Luca *et al.,* 2012.) **(B)** PCR with the *Tylenchulus* species-specific primers. Lanes: M, 100 bp DNA marker (Promega); 1, *Tylenchulus musicola*; 2, *T. semipenetrans*; 3, *T. graminis*; 4, *T. furcus*; 5, *T. palustris*; 6, control without DNA. (After Tanha Maafi *et al*., 2012.)

12.4.1 Protocols for PCR

Protocol 1: PCR with Taq PCR Core Kit (Qiagen) for amplification of rRNA and protein-coding genes (Subbotin *et al*., 2018)

- Add 1–4 µl of extracted DNA to a 0.2 ml Eppendorf tube containing 2.5 µl 10× PCR buffer, 5 µl Q solution, 0.5 µl dNTPs mixture (10 mM each) (*Taq* PCR Core Kit, Qiagen), 0.15 µl of each primer (1.0 µg μl^{-1}), 0.1 µl *Taq* Polymerase, and distilled water to a final volume of 25 µl.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 94°C for 4 min, 35 cycles of 1 min at 94°C, 1 min 30 s at 55°C, and 2 min at 72°C, followed by a final step of 10 min at 72°C.

Protocol 2: PCR with DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific) for amplification of mitochondrial gene (Subbotin *et al*., 2018)

- Add 1–4 µl of extracted DNA to a 0.2 ml Eppendorf tube containing 10 µl DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific), 10 µl water and 0.15 µl of each primer (1.0 µg μl^{-1}), and distilled water to a final volume of 25 µl.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 94°C for 4 min, followed by 40 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min 30 s at 72°C, with a final extension at 72°C for 10 min.

Protocol 3: PCR with Apex Taq RED DNA Polymerase Master Mix (2×) (Genesee Scientific) for amplification of ribosomal RNA and mitochondrial gene (Ye *et al*., 2015)

- Add 1 µl of extracted DNA to a 0.2 ml Eppendorf tube containing 12.5 µl 2× Apex Taq red master mix DNA polymerase (Genesee Scientific), 9.5 µl water, 1 µl each of 10 µM forward and reverse primers.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 95°C for 5 min, followed by 40 cycles of 30 s at 94°C, 45 s at 55°C, and 1 min at 72°C, with a final extension at 72°C for 10 min.

Protocol 4: PCR with HOT FIREPol® (Solis BioDyne) for amplification of rRNA and protein-coding genes (Archidona-Yuste *et al*., 2018)

- Add 1–4 µl of extracted DNA to a 0.2 ml Eppendorf tube containing 5 µl of 5× HOT FIREpol® Blend Master Mix (with 10 mM $MgCl_2$), 0.15 µl of each primer (1.0 µg $µl^{-1}$), and distilled water to a final volume of 25 µl.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 95°C for 15 min, 35 cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C, followed by a final step of 5 min at 72°C. For some specific A + T rich gene region as the *coxII*-16S used for identification of *Meloidogyne* species: an initial denaturation at 95°C for 15 min, 40 cycles of 1 min at 95°C, 1 min at 54°C, and 2 min 30 s at 66°C, followed by a final step of 7 min at 68°C.

Protocol 5: PCR with TaKaRa Ex Taq (Takara Bio) for amplification of rRNA (Carta and Li, 2018)

- Add 4 µl of extracted DNA to 0.2 ml Eppendorf tube containing 5 µl 10× Ex Taq Buffer, 4 µl dNTP mixture (2.5 mM each), 1 µl of each primer (10 µM), 0.25 µl of TaKaRa Ex Taq (5 units $µl^{-1}$) and 34.75 µl of distilled water.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 95°C for 3 min, 5 cycles of 30 s at 94 °C, 40 s at 45°C, 2 min at 72°C, 40 cycles of 30 s at 94°C, 40 s at 55°C, 2 min at 72 °C, followed by a final step of 5 min at 72°C.

Protocol 6: PCR with Phusion Taq (BioLabs) for amplification of rRNA (Carta and Li, 2018)

- Add 4 µl of extracted DNA to a 0.2 ml Eppendorf tube containing 10 µl of 5× Phusion HF Buffer, 1 µl of 10 mM dNTPs, 2.5 µl of each 10 µM primer, 0.5 µl of Phusion DNA polymerase and distilled water added up to 29.5 µl. It is recommended that all reaction components are assembled on ice and quickly transferred to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed and centrifuged prior to use. It is important to add Phusion DNA polymerase last in order to prevent any primer degradation.
- Put the tube in the PCR machine with the following thermal profile: an initial denaturation at 98°C for 30 s, 35 cycles of 10 s at 98°C, 30 s at 59°C, 90 s at 72°C followed by a final step of 5 min at 72°C.

12.4.2 Gel electrophoresis

Pour 100 ml of 1x TAE into a clean 250 ml flask. Add 1 g of agarose to make a 1% gel; microwave until the solution is clear. Pour the warm liquid agarose into a gel casting tray. Wait until the gel polymerizes. Run 2–5 µl of PCR product on an agarose gel for 30–60 min at 90–100 V. Put the unstained gel in a container and pour some TAE buffer and EtBr solution. Incubate with mild shaking. DNA-staining dye (GelRed® or GelGreen®) could be added directly in an agarose gel instead of staining in EtBr solution. Use the UV transilluminator to visualize the DNA bands.

12.5 PCR-Restriction Fragment Length Polymorphism

Variation in sequences in PCR products can be revealed by restriction endonuclease digestion. The PCR product obtained from different species or populations can be digested by a restriction enzyme, after which the resulting fragments are separated by electrophoresis. If differences in fragment length occur within restriction sites, the digestion of the PCR products will yield restriction fragment length polymorphism (RFLP), i.e. different RFLP profiles. PCR-RFLP of the ITS region of the rRNA gene is a very reliable method for identification of many plant-parasitic nematode groups including cyst forming, root-knot, lesion, stem, gall forming and longidorids (Fig. 12.3), as well as nematodes from the genera *Bursaphelenchus* and *Aphelenchoides*. Six to nine restriction enzymes enable most of the economical important species of cyst forming nematodes to be distinguished from each other as well as from their sibling species. RFLP of the ITS-rDNA obtained after restriction with several enzyme combinations enables identification of important root-knot nematode species; however, it fails to separate species from the tropical group, including *M. javanica*, *M. incognita* and *M. arenaria*. PCR-RFLP of mtDNA fragments between cytochrome oxidase *COII* gene and 16S has been successfully applied for diagnostics of these nematodes.

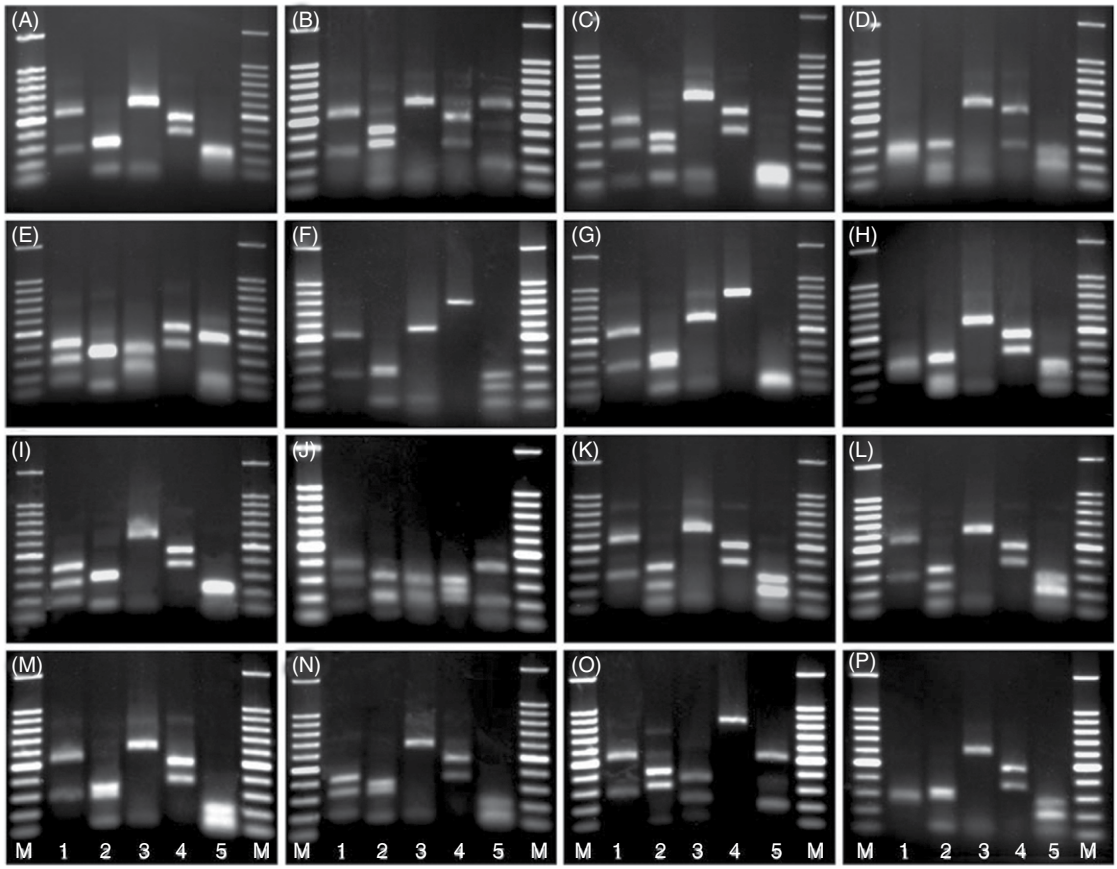

Fig. 12.3. PCR-D2-D3-28S-RFLP diagnostic profiles for some *Longidorus* species. **(A)** *L. aetnaeus*. **(B)** *L. africanus*. **(C)** *L. americanus*. **(D)** *L. artemisiae*. **(E)** *L. caespiticola* type B. **(F)** *L. elongatus*. **(G)** *L. euonymus*. **(H)** *L. intermedius*. **(I)** *L. leptocephalus*. **(J)** *L. lignosus*. **(K)** *Longidorus* sp. 1. **(L)** *Longidorus* sp. 2. **(M)** *Longidorus* sp. 3. **(N)** *Longidorus* sp. 4. **(O)** *Longidorus* sp. 5. **(P)** *Longidorus* sp. 6. Lanes: M, 100 bp DNA marker (Promega); 1, *Alu*I; 2, *Hinf*I; 3, *Bsp*143I; 4, *Tru*1I; 5, *Rsa*I. (After Subbotin *et al.*, 2013.)

12.6 Real-time PCR

DNA technology also provides several methods for quantification of nematodes in samples. Real-time PCR requires an instrumentation platform that consists of a thermal cycler, optics for fluorescence excitation and emission collection, and computerized data acquisition and analysis software (Fig. 12.4A,B). Real-time PCR is the continuous collection of fluorescent signals from one or more polymerase chain reactions over a range of cycles. The real-time technique allows monitoring of the sample during PCR using hybridization probes (TaqMan, Molecular Beacons, and Scorpions) or double-stranded dyes, such as SYBR Green, resulting in an increase in fluorescence signal. The amplification of any template is defined by four phases: (i) baseline; (ii) exponential; (iii) linear; and (iv) plateau. Quantitative PCR requires the measurement to be taken before the plateau phase, so the relationship between the number of cycles and molecules is relatively linear. The length of exponential phase depends on the template concentration and the quality of the real-time assay. Quantitative real-time PCR is the conversion of the fluorescent signals from each reaction into a numerical value for each sample. Real-time PCR instruments use for calculations the cycle threshold (C_t) or the point when the level of fluorescence exceeds some arbitrary threshold. A plot of cycle number

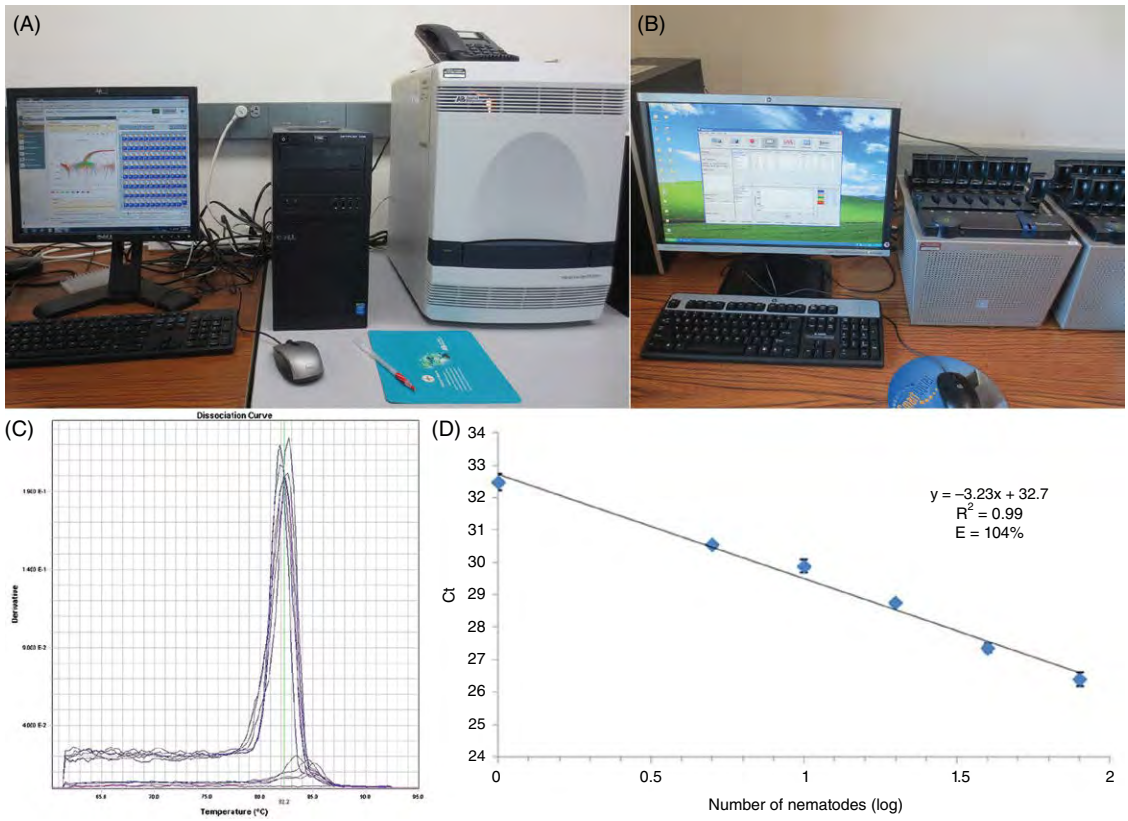

Fig. 12.4. **(A)** Applied Biosystems 7500 Real-Time PCR System. **(B)** SmartCycler Thermal Cycler Automated Real-Time PCR System. **(C)** Dissociation curve of the qPCR test (SensiFAST SYBR Hi-ROX) with annealing temperature set at 62°C showing high peaks at ± 82.2°C of two *Pratylenchus penetrans* populations (n = 3) and minor peaks for a population of *P. thornei* (n = 3) and NTC (n = 2). **(D)** Standard curve of the qPCR assay (SensiFAST Probe Hi-ROX) for *P. penetrans*: threshold cycle number (Ct) plotted against the log of the number of individuals of *P. penetrans* (1, 5, 10, 20, 40 and 80). (After Mokrini *et al.*, 2013.)

versus a log scale of the DNA concentration should result in a linear relationship during the exponential phase of PCR amplification.

SYBR Green binds only to double-stranded DNA and becomes fluorescent only when bound. This dye has the virtue of being easy to use because it has no sequence specificity and it can be used to detect any PCR product. However, this virtue has a drawback, as the dye binds also to any non-specific product, including primer dimers. To overcome this problem, the melting curve analysis can be employed. The products of PCR reaction are melted by increasing the temperature of the sample (Fig. 12.4C). The non-specific product tends to melt at a much lower temperature than the longer specific product. Both the shape and position of the DNA melting curve area are a function of the GC/AT ratio. The length of amplicon can be used to differentiate amplification products separated by less than 2°C in *T*m (the melting temperature).

The disadvantage of using a fluorescent dye is that it binds to any double-stranded DNA and then it cannot be used for quantification of several targets in a multiplex real-time PCR because it cannot distinguish between different sequences. In this case, sequence-specific fluorescent probes, such as TaqMan probes, are employed.

The rise in fluorescence is correlated to the initial DNA template amounts when compared with samples of known DNA concentration. Several DNA samples with known concentrations are used to generate a

standard curve based on their measured C_t values. A sample with an unknown DNA quantity can be compared to this standard curve to calculate its initial DNA template concentration (Fig. 12.4D). The PCR quantification technique measures the number of nematodes indirectly by assuming that the amount of target DNA concentration (copies) in the sample is proportional to the number of targeted nematodes.

Several real-time PCR assays for detection and quantification of different plant-parasitic nematodes have been published and are briefly reviewed by Braun-Kiewnick and Kiewnick (2018). Compared with the traditional PCR method, real-time PCR has several advantages. It allows for faster, simultaneous detection and quantification of target DNA. The automated system overcomes the laborious process of estimating the quantity of the PCR product after gel electrophoresis and results can be seen in real-time. PCR assays that can be easily adapted for high-throughput analyses of many samples at a time – 96 or 384 formats.

Protocol 1: Detection and quantification of *Pratylenchus penetrans* using SensiFAST SYBR Hi-ROX (after Mokrini *et al*., 2013)

- The reaction tube contains 20 µl reaction mixture with 10 µl of SensiFAST SYBR Hi-ROX (2×), 400 nM of each primer (PpenMFor 3′-CCA ACC TCT GCT ACA CTA-5′ and PpenMRev 3′-CAG TGC CGT ATT CAG TGA-5′), 200 nM of the probe (PpMPb 3′-CAC TAT GCC GC-5′, labelled with 6-FAM) and 3 µl of DNA template.
- The amplification program consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 63°C for 30 s and 72°C for 1 min.

Protocol 2: Detection and quantification of *Meloidogyne hapla* using Bio Probe Mix LoRox (Sapkota *et al*., 2016)

- The tube contains a reaction mixture of 13 µl with 0.9 µM of each of primer (Mhaplafwd 5′-TGG TTC AGG GTC ATT TTT CTA TAA AGT-3′ and Mhaplarev 5′-CAA ATC GCT GCG TAC CAA CA-3′), 0.25 µM of probe (Mhapla MGB Probe 5′-FAM-CCA TTG GCA CTA TAA C-MGB-3′) and 7.5 µl of Bio Probe Mix LoRox (PCR Biosystems). Water and 2 µl of DNA template are added to a total volume of 15 µl.
- The amplification program consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min.

12.7 Problems with PCR-based Methods

The application of conventional PCR with universal and specific primers for diagnostic purposes has revealed several limitations. First, so-called universal primers, which are designed based on existing knowledge of DNA sequences for target species, might not work for some other species that have different sequences in this target DNA region. Second, universal primers presently available for application in diagnostics can also amplify organisms other than nematodes, such as fungi and plants.

Application of PCR with a specific primer could face similar problems. One limitation is the potential for obtaining a false positive reaction. As a specific primer design is always based on analysis of DNA sequences for a target species and closely related nematodes, there is a possibility that similar fragments can be obtained for another, previously non-investigated and tested, nematode species. A second limitation is the possibility of a false negative reaction. Although a region with a conserved sequence should be used for primer design, the possibility cannot be excluded that some mutations might have occurred in this region in some specimens or populations of the target taxon. As a result, such specimens or populations might not be detectable by the PCR test. Another limitation that should be always considered is the possibility of sample contamination, which might occur during sample preparation in a laboratory. This might give a false positive reaction due to the great sensitivity of the PCR method with a specific primer. Following strict rules to prevent contamination during preparation of the PCR mixture and adding a control tube without DNA in the PCR test is imperative for all diagnostics tests.

12.8 PCR Purification

Purification of DNA from a PCR reaction is necessary for sequencing, and facilitates the removal of enzymes, nucleotides, primers and buffer components. Commonly used methods employ spin columns containing a silica membrane, which binds DNA under specific salt concentrations and the remaining sample is washed

out. These are performed during the successive wash steps. DNA elution can then be performed in either Tris buffer or distilled water. The PCR product can be cleaned using QIAquick PCR Purification Kit (Quagen), DNA Clean & Concentrator-5 (Zymo Research) or kits provided by other companies. Another approach is PCR product cleaning with enzyme digestion. ExoSAP-IT™ (Applied Biosystems) PCR Product Cleanup Reagent is used for enzymatic cleanup of the amplified PCR product. It hydrolyses single-stranded DNA, primers and nucleotides in a single step. The reaction setup is complete with one pipetting step, which is followed by two incubations. The first incubation digests excess primer and dephosphorylates nucleotides. The second, high temperature incubation inactivates the enzymes.

12.9 Cloning of PCR Products

In a genome, rRNA and protein-coding genes are present in many copies and, although their sequences are often identical, some copies may have sequence variations. PCR amplification of such gene fragments yields a complex mixture with a product containing copies with different sequences. If some highly abundant copies have insertions/deletions, often the PCR product cannot be directly sequenced and should be cloned. Cloning is a method in which double-stranded DNA fragments amplified by PCR are ligated directly into a vector. The vector transports the DNA fragment into a bacterial host cell. Within the host cell the vector multiplies, producing numerous identical copies of the recombinant DNA. After a large number of cell divisions, a colony, or clone, of identical host cells is produced. Each cell in the clone contains one copy of the recombinant DNA molecule. PCR of colonies enables an amplicon from a single copy to be obtained and then this product can be submitted for sequencing.

The cloning technique requires a small amount of starting template materials and, thus, poorly amplified PCR product that failed for direct sequencing can be submitted for cloning to get high-quality DNA sequencing results from its clones. The cloning of DNA fragments essentially involves several steps: (i) preparation and purification of target DNA; (ii) preparation of vector DNA; (iii) creation of recombinant DNA; (iv) introduction of recombinant DNA into bacteria; and (v) selection of bacterial clones containing recombinant DNA. Cloning kits are provided by many companies, including Promega (pGEM®-T Easy Vector Systems, pGEM®-T Vector Systems), Qiagen (QIAGEN PCR Cloning Kit) (see: https://www.promega.com/-/media/files/resources/education-and-training/unit-6/sm0060710.pdf).

12.10 DNA Sequencing

The process of determining the order of the nucleotide bases along a DNA strand is called sequencing. Nucleic acid sequencing methods have undergone tremendous advances over the past decade. The rRNA, mtDNA and other gene sequences have been determined for a large number of nematode species and have been deposited into the GenBank database (http://www.ncbi.nlm.nih.gov/). In general, the comparison of the genes with reference data using sequence and phylogenetic analysis enables the identification of nematode samples.

12.11 References

Al-Banna, L., Ploeg, A.T., Williamson, V.M. and Kaloshian, I. (2004) Discrimination of six *Pratylenchus* species using PCR and species-specific primers. *Journal of Nematology* 36, 142–146.

Amiri, S., Subbotin, S.A. and Moens, M. (2002) Identification of the beet cyst nematode *Heterodera schachtii* by PCR. *European Journal of Plant Pathology* 108, 497–506. DOI: 10.1023/A:1019974101225

Archidona-Yuste, A., Cantalapiedra Navarrete, C., Liébanas, G., Rapoport, H.F., Castillo, P. and Palomares-Rius, J.E. (2018) Diversity of root-knot nematodes of the genus *Meloidogyne* Goëldi, 1892 (Nematoda: Meloidogynidae) associated with olive plants and environmental cues regarding their distribution in southern Spain. *PLoS One* 13(6): e0198236 DOI: 10.1371/journal.pone.0198236

Atkins, S.D., Manzanilla-López, R.H., Franco, J., Peteira, B. and Kerry, B.R. (2005) A molecular diagnostic method for detecting *Nacobbus* in soil and in potato tubers. *Nematology* 7, 193–202. DOI: 10.1163/1568541054879539.

Bae, C.H., Robbins, R.T. and Szalanski, A.L. (2009) Molecular identification of some *Hoplolaimus* species from the USA based on duplex PCR, multiplex PCR and PCR-RFLP analysis. *Nematology* 11, 471–480. DOI: 10.1163/156854109X447042

Baidoo, R., Yan, G.P., Nagachandrabose, S. and Skantar, A.M. (2017) Developing a Real-time PCR assay for direct identification and quantification of *Pratylenchus penetrans* in soil. *Plant Disease* 101, 1432–1441. DOI: 10.1094/PDIS-01-17-0117-RE

Bhadury, P., Austen, C.M., Bilton, D.T., Lambshead, J.D., Rogers, A.D. and Smerdon, G.R. (2005) Combined morphological and molecular analysis of individual nematodes through shortterm preservation in formalin. *Molecular Ecology Notes* 5, 965–968. DOI: 10.1111/j.1471-8286.2005.01095.x

Bhadury, P., Austen, M.C., Bilton, D.T., Lambshead, P.J.D., Rogers, A.D. and Smerdon, G.R. (2007) Exploitation of archived marine nematodes – a hot lysis DNA extraction protocol for molecular studies. *Zoologica Scripta* 36, 93–98. DOI: 10.1111/j.1463-6409.2006.00250.x

Blaxter, M.L., De Ley, P., Garey, J.R. *et al*. (1998) A molecular evolutionary framework for the phylum Nematoda. *Nature* 392, 71–75. DOI: 10.1038/32160

Blok, V.C., Phillips, M.S., McNicol, J.W. and Fargette, M. (1997) Genetic variation in tropical *Meloidogyne* spp. as shown by RAPDs. *Fundamental and Applied Nematology* 20, 127–133.

Braun-Kiewnick, A. and Kiewnick, S. (2018) Real-time PCR, a great tool for fast identification, sensitive detection and quantification of important plant-parasitic nematodes. *European Journal of Plant Pathology* 152, 271–283. DOI: 10.1007/s10658-018-1487-7

Bulman, S.R. and Marshall, J.W. (1997) Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). *New Zealand Journal of Crop and Horticultural Science* 25, 123–129.

Cantalapiedra-Navarrete, C., Navas-Cortés, J.A., Liébanas, G., Vovlas, N., Subbotin, S.A., Palomares-Rius, J.E. and Castillo, P. (2013) Comparative molecular and morphological characterisations in the nematode genus *Rotylenchus*: *Rotylenchus paravitis* n. sp., an example of cryptic speciation. *Zoologischer Anzeiger* 252, 246–268. DOI: 10.1016/j.jcz.2012.08.002

Carrasco-Ballesteros, S., Castillo, P., Adams, B.J. and Perez-Artes, E. (2007) Identification of *Pratylenchus thornei*, the cereal and legume root-lesion nematode, based on SCAR-PCR and satellite DNA. *European Journal of Plant Pathology* 118, 115–125. DOI: 10.1007/s10658-007-9110-3

Carta, L.K. and Li, S. (2018) Improved 18S small subunit rDNA primers for problematic nematode amplification. *Journal of Nematology* 50, 533–542. DOI: 10.21307/jofnem-2018-051

Carta, L.K. and Li, S. (2019) PCR amplification of a long rDNA segment with one primer pair in agriculturally important nematodes. *Journal of Nematology* 51, 1–8. DOI: 10.21307/jofnem-2019-026

Caswell-Chen, E.P., Williamson, V.M. and Wu, F.F. (1992) Random amplified polymorphic DNA analysis of *Heterodera cruciferae* and *H. schachtii* populations. *Journal of Nematology* 24, 342–351.

Chase, M.T., Etter, R.J., Rex, A. and Quattro, J.M. (1998) Extraction and amplification of mitochondrial DNA from formalin-fixed deep-sea molluscs. *Biotechniques* 24, 243–247. DOI: 10.2144/98242bm16

Chen, F.M., Negi, S. and Ye, J.R. (2011) A SCAR molecular marker to distinguish *Bursaphelenchus mucronatus* from the pinewood nematode, *B. xylophilus. Forest Pathology* 41, 376–381. DOI: 10.1111/j.1439-0329.2010.00693.x

Chizhov, V.N., Chumakova, O.A., Subbotin, S.A. and Baldwin, J.G. (2006) Morphological and molecular characterization of foliar nematodes of the genus *Aphelenchoides: A. fragariae* and *A. ritzemabosi* (Nematoda: Aphelenchoididae) from the Main Botanical Garden of the Russian Academy of Sciences, Moscow. *Russian Journal of Nematology* 14, 179–184.

Chizhov, V.N., Butorina, N.N. and Subbotin, S.A. (2012) Entomoparasitic nematodes of the genus *Skarbilovinema*: *S. laumondi* and *S. lyoni* (Nematoda: Tylenchida), parasites of the flies of the family Syrphidae (Diptera) with phylogeny of the suborder Hexatylina. *Russian Journal of Nematology* 20, 141–155.

Chizhov, V.N., Pridannikov, M.V., Peneva, V. and Subbotin, S.A. (2014) Morphological and molecular characterisation of the Saratov population of the European dagger nematode, *Xiphinema diversicaudatum* (Nematoda: Dorylaimida), with notes on phylogeography of the species. *Nematology* 16, 847–862. DOI: 10.1163/15685411-00002813

Consoli, E.A., Oliveira, S.A., Harakava R. and Oliveira, C.M.G. (2012) [Development of a molecular diagnostic for the identification of *Pratylenchus jaehni*.] *Nematologia Brasileira* 36, 62–70.

Cui, R.Q., Zhao, L.R. and Zhong, G.Q. (2010) [A rapid method to detect *Aphelenchoides ritzemabosi* by PCR.] *Acta Agriculturae Universitatis Jiangxiensis* 21, 714–717.

Curran, J., Baillie, D.L. and Webster, J.M. (1985) Use of restriction fragment length differences in genomic DNA to identify nematode species. *Parasitology* 90, 137–144. DOI: 10.1017/S0031182000049088

Curran, J., Driver, F., Ballard, J.W.O. and Milner, R.J. (1994) Phylogeny of *Metarhizium*: analysis of ribosomal DNA sequence data. *Mycological Research* 98, 547–555. DOI: 10.1016/S0953-7562(09)80478-4

De Luca, F., Troccoli, A., Duncan, L.W., Subbotin, S.A., Waeyenberge, L., Coyne, D.L., Brentu, F.C. and Inserra, R.N. (2012) *Pratylenchus speijeri* n. sp. (Nematoda: Pratylenchidae), a new root-lesion nematode pest of plantain in West Africa. *Nematology* 14, 987–1004. DOI: 10.1163/156854112X638424

Devran, Z., Tülek, A., Mıstanoğlu, I., Çiftçiğil, T.H. and Özalp, T. (2017) A rapid molecular detection method for *Aphelenchoides besseyi* from rice tissues. *Australasian Plant Pathology* 46, 43–48. DOI: 10.1007/s13313-016-0452-1

Dong, K., Dean, R.A., Fortnum, B.A. and Lewis, S.A. (2001) Development of PCR primers to identify species of root-knot nematodes: *Meloidogyne arenaria*, *M. hapla*, *M. incognita* and *M. javanica. Nematropica* 31, 271–280.
Esquibet, M., Grenier, E., Plantard, O., Andaloussi, F.A. and Caubel, G. (2003) DNA polymorphism in the stem nematode *Ditylenchus dipsaci*: Development of diagnostic markers for normal and giant races. *Genome* 46, 1077–1083. DOI: 10.1139/g03-072
Ferris, V.R., Ferris, J.M. and Faghihi, J. (1993) Variation in spacer ribosomal DNA in some cystforming species of plant parasitic nematodes. *Fundamental and Applied Nematology* 16, 177–184.
Filipiak, A., Jakubowska, A. and Tomalak, M. (2010) Species-specific polymerase chain reaction primers for simple detection of *Bursaphelenchus fraudulentus* (Nematoda: Parasitaphelenchidae). *Nematology* 12, 157–160. DOI: 10.1163/138855409X12469543767319
Floyd, R., Abebe, E., Papert, A. and Blaxter, M. (2002) Molecular barcodes for soil nematode identification. *Molecular Ecology* 11, 839–850. DOI: 10.1046/j.1365-294X.2002.01485.x
Floyd, R.M., Rogers, A.D., Lambshead, J.D. and Smith, C.R. (2005) Nematode-specific PCR primers for the 18S small subunit rRNA gene. *Molecular Ecology Note* 5, 611–612. DOI: 10.1111/j.1471-8286.2005.01009.x
Goto, K., Sato, E. and Toyota, K. (2009) A novel detection method for the soybean cyst nematode *Heterodera glycines* using soil compaction and real-time PCR. *Japanese Journal of Nematology.* 39, 1–7.
Holterman, M., van der Wurff, A., van den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., Bakker, J. and Helder, J. (2006) Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades. *Molecular Biology and Evolution* 23, 1792–1800. DOI: 10.1093/molbev/msl044
Huang, D. and Yan, D. (2017) Detection of the root-lesion nematode *Pratylenchus scribneri* using conventional and real-time PCR. *Plant Disease* 101, 359–365. DOI: 10.1094/PDIS-07-16-1013-RE
Hübschen, J., Kling, L., Ipach, U., Zinkernagel, V., Brown, D.J.F. and Neilson R. (2004) Development and validation of species-specific primers that provide a molecular diagnostic for virus-vector longidorid nematodes and related species in German viticulture. *European Journal of Plant Pathology* 110, 883–891. DOI: 10.1007/s10658-004-4841-x
Jeszke, A., Dobosz, R. and Obrępalska-Stęplowska, A. (2015) A fast and sensitive method for the simultaneous identification of three important nematode species of the genus *Ditylenchus. Pest Management Science* (2015) 71, 243–249. Epub 2014 May 8. DOI: 10.1002/ps.3788
Jiang, L., Zheng, J.W., Waeyenberge, L., Subbotin, S.A. and Moens, M. (2005) Duplex PCR based identification of *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) Nickle, 1970. *Russian Journal of Nematology* 13, 115–121.
Kerkoud, M., Esquibet, M. and Plantard, O. (2007) Identification of *Ditylenchus* species associated with Fabaceae seeds based on a specific polymerase chain reaction of ribosomal DNA-ITS regions. *European Journal of Plant Pathology* 118, 323–332. DOI: 10.1007/s10658-006-9092-6
Ko, H.R., Kim, E.H., Kim, S.J., Lee, J.K. and Lee, W.H. (2017) Rapid methods to distinguish *Heterodera schachtii* from *Heterodera glycines* using PCR technique. *Research in Plant Disease* 23, 241–248. DOI: 10.5423/RPD.2017.23.3.241
Liu, B., Mei, Y. and Zheng, J. (2007) [Species-specific detection of inter-populations of *Ditylenchus destructor*.] *Journal of Zhejiang University (Agriculture and Life Science)* 33, 490–496.
Liu, Y.F., Xu, C.L., Zhang, C., Su, X.M. and Xie, H. (2011) Direct PCR detection of *Radopholus similis* from plant tissues and mixed sample of nematodes by ITS specific primers. *Scientia Agricultura Sinica* 44, 3991–3998.
Long, H., Liu, H. and Xu, J.H. (2006) Development of a PCR diagnostic for the root-knot nematode *Meloidogyne enterolobii*. *Acta Phytopathologica Sinica* 36, 109–115.
Machado, A.C.Z., Ferraz, L.C.C.B. and Oliveira, C.M.G. (2007) Development of a species-specific reverse primer for the molecular diagnostic of *Pratylenchus brachyurus. Nematropica* 37, 249–257.
Madani, M., Tenuta, M., Chizhov, V.N. and Subbotin, S.A. (2015) Diagnostics of stem and bulb nematodes, *Ditylenchus weischeri* and *D. dipsaci* (Nematoda: Anguinidae), using PCR with species-specific primers. *Canadian Journal of Plant Pathology* 37, 212–220. DOI: 10.1080/07060661.2015.1035754
Matsunaga, K. and Togashi, K. (2004) A simple method for discriminating *Bursaphelenchus xylophilus* and *B. mucronatus* by species-specific polymerase chain reaction primer pairs. *Nematology* 6, 273–277. DOI: 10.1163/1568541041217960
McCuiston, J.L., Hudson, L.C., Subbotin, S.A., Davis, E.L. and Warfield, C.Y. (2007) Conventional and PCR detection of *Aphelenchoides fragariae* in diverse ornamental host plant species. *Journal of Nematology* 39, 343–355.
Mekete, T., Reynolds, K., Lopez-Nicora, H.D., Gray, M.E. and Niblack, T.L. (2011) Distribution and diversity of root-lesion nematode (*Pratylenchus* spp.) associated with *Miscanthus* × *giganteus* and *Panicum virgatum* used for biofuels, and species identification in a multiplex polymerase chain reaction. *Nematology* 13, 673–686. DOI: 10.1163/138855410X538153

Meng, Q.P., Long, H. and Xu, J.H. (2004) PCR assays for rapid and sensitive identification of three major root-knot nematodes*, Meloidogyne incognita*, *M. javanica* and *M. arenaria. Acta Phytopathologica Sinica* 34, 204–210.

Mokrini, F., Waeyenberge, L., Viaene, N., Andaloussi, F.A. and Moens, M. (2013) Quantitative detection of the root-lesion nematode, *Pratylenchus penetrans*, using qPCR. *European Journal of Plant Pathology* 137, 403–413. DOI: 10.1007/s10658-013-0252-1

Mulholland, V., Carde, L., O'Donnell, K.L., Fleming, C.C. and Powers, T.O. (1996) Use of the polymerase chain reaction to discriminate potato cyst nematode at the species level. In: Marshall, C. (ed.) *Proceedings of Diagnostics in Crop Production Symposium*. British Crop Production Council, Farnham, UK, pp. 247–252.

Nunn, G.B. (1992) *Nematode Molecular Evolution*. Ph.D. Thesis. University of Nottingham, Nottingham, UK.

Ou, S., Peng, D., Liu, X., Li, Y. and Moens, M. (2008) Identification of *Heterodera glycines* using PCR with sequence characterised amplified region (SCAR) primers. *Nematology* 10, 397–403. DOI: 10.1163/156854108783900212

Palomares-Rius, J.E., Guesmi, I., Horrigue-Raouani, N., Cantalapiedra-Navarrete, C., Liébanas, G. and Castillo, P. (2014) Morphological and molecular characterisation of *Pratylenchus oleae* n. sp. (Nematoda: Pratylenchidae) parasitizing wild and cultivated olives in Spain and Tunisia. *European Journal of Plant Pathology* 140, 53–67. DOI: 10.1007/s10658-014-0443-4

Peng, H., Qi, X., Peng, D., Long, H., He, X., Huang, W. and He, W. (2013) Sensitive and direct detection of *Heterodera filipjevi* in soil and wheat roots by species-specific SCAR-PCR assays. *Plant Disease* 97, 1288–1294. DOI: 10.1094/PDIS-02-13-0132-RE

Randig, O., Bongiovanni, M., Carneiro, R.M.D.G., Sarah, J.-L. and Castagnone-Sereno, P. (2002) A species-specific satellite DNA family in the genome of the coffee root-knot nematode *Meloidogyne exigua*: application to molecular diagnostics of the parasite. *Molecular Plant Pathology* 3, 431–437. DOI:10.1046/j.1364-3703.2002.00134.x

Ravindran, A., Kumar, A., Antony, D. and Eapen, S.J. (2011) Single tube duplex PCR for simultaneous detection of *Phytophthora capsici* and *Radopholus similis* infecting black pepper (*Piper nigrum*). *Indian Phytopathology* 64, 353–357.

Riga, E., Karanastasi, E., Oliveira, C.M.G. and Neilson, R. (2007) Molecular identification of two stubby root nematode species. *American Journal of Potato Research* 84, 161–167. DOI: 10.1007/BF02987139

Rubtsova, T.V., Moens, M. and Subbotin, S.A. (2005) PCR amplification of a rRNA gene fragment from formalin-fixed and glycerine-embedded nematodes from permanent slides. *Russian Journal of Nematology* 13, 137–140.

Sapkota, R., Skantar, A.M. and Nicolaisen, M. (2016) A TaqMan real-time PCR assay for detection of *Meloidogyne hapla* in root galls and in soil. *Nematology* 18, 147–154. DOI: 10.1163/15685411-00002950

Sato, E., Goto, K., Min, Y.Y., Toyota, K. and Suzuki, C. (2010) Quantitative detection of *Pratylenchus penetrans* from soil using soil compaction and real-time PCR. *Nematology Research* 40, 1–6.

Sayler, R.J., Walker, C., Goggin, F., Agudelo, P. and Kirkpatrick, T. (2012) Conventional PCR detection and real-time PCR quantification of reniform nematodes. *Plant Disease* 96, 1757–1762. DOI: 10.1094/PDIS-12-11-1033-RE

Silva, A.P., Parra, P.P, Campos, V.P., Costa, S.S., Vicente, C.S.L., Ferreira, L.G., Souza, R.M. and Mota, M. (2016) Genetic diversity of *Bursaphelenchus cocophilus* in South America. *Nematology* 18, 605–614. DOI: 10.1163/15685411-00002980

Subbotin, S.A., Peng, D. and Moens, M. (2001) A rapid method for the identification of the soybean cyst nematode *Heterodera glycines* using duplex PCR. *Nematology* 3, 365–370. DOI: 10.1163/156854101317020286

Subbotin, S.A., Madani, M., Krall, E., Sturhan, D. and Moens, M. (2005) Molecular diagnostics, taxonomy, and phylogeny of the stem nematode *Ditylenchus dipsaci* species complex based on the sequences of the internal transcribed spacer-rDNA. *Phytopathology* 95, 1308–1315. DOI: 10.1094/PHYTO-95-1308

Subbotin, S.A., Rogozhin, E.A. and Chizhov, V.N. (2013) Molecular characterisation and diagnostics of some *Longidorus* species (Nematoda: Longidoridae) from Russia and other countries using rRNA genes. *European Journal of Plant Pathology* 138, 377–390. DOI: 10.1007/s10658-013-0338-9

Subbotin, S.A., Toumi, F., Elekçioğlu, I.H., Waeyenberge, L. and Tanha Maafi, Z. (2018) DNA barcoding, phylogeny and phylogeography of the cyst nematodes of the *Avenae* group from the genus *Heterodera* (Tylenchida: Heteroderidae) *Nematology* 20, 671–702. DOI: 10.1163/15685411-00003170

Szalanski, A.L., Sui, D.D., Harris, T.S. and Powers, T.O. (1997) Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. *Journal of Nematology* 29, 255–267.

Tanha Maafi, Z., Amani, M., Stanley, J.D., Inserra, R.N., Van den Berg, E. and Subbotin, S.A. (2012) Description of *Tylenchulus musicola* sp. n. (Nematoda: Tylenchulidae) from banana in Iran with molecular phylogeny and characterization of species of *Tylenchulus* Cobb, 1913*. Nematology* 14, 353–369. DOI: 10.1163/156854111X596242

Thomas, W.K., Vida, J.T., Frisse, L.M., Mundo, M. and Baldwin, J.G. (1997) DNA sequences from formalin-fixed nematodes: integrating molecular and morphological approaches to taxonomy. *Journal of Nematology* 29, 250–254.

Toumi, F., Waeyenberge, L., Viaene, N., Dababat, A.A., Nicol, J.M., Ogbonnaya, F. and Moens, M. (2013a) Development of two species-specific primer sets to detect the cereal cyst nematodes *Heterodera avenae* and *Heterodera filipjevi. European Journal of Plant Pathology* 136, 613–624. DOI: 10.1007/s10658-013-0192-9

Toumi, F., Waeyenberge, L., Viaene, N., Dababat, A.A., Nicol, J.M., Ogbonnaya, F. and Moens, M. (2013b) Development of a species-specific PCR to detect the cereal cyst nematode, *Heterodera latipons. Nematology* 15, 709–717. DOI: 10.1163/15685411-00002713

Troccoli, A., Subbotin, S.A., Chitambar, J.J., Janssen, T, Waeyenberge L., Stanley, J.D., Duncan L.W., Agudelo, P., Munera Uribe, G., Franco, J. and Inserra, R.N. (2016) Characterisation of amphimictic and parthenogenetic populations of *Pratylenchus bolivianus* Corbett, 1983 (Nematoda: Pratylenchidae) and their phylogenetic relationships with closely related species. *Nematology* 18, 651–678. DOI: 10.1163/15685411-00002981

Uehara, T., Mizukubo, T., Kushida, A. and Momota, Y (1998) Identification of *Pratylenchus penetrans* (Cobb) by PCR using ITS-based species-specific primers. *Japanese Journal of Nematology* 28, 1–7.

Van den Berg, E., Tiedt, L.R., Coyne, D.L., Ploeg, A.T., Navas-Cortés, J.A., Roberts, P.A., Yeates, G.W. and Subbotin, S.A. (2013) Morphological and molecular characterisation and diagnostics of some species of *Scutellonema* Andrássy, 1958 (Tylenchida: Hoplolaimidae) with a molecular phylogeny of the genus. *Nematology* 15, 719–745. DOI: 10.1163/15685411-00002714

Van den Berg, E., Palomares-Rius, J.E., Vovlas, N., Tiedt, L.R., Castillo, P. and Subbotin, S.A. (2016) Morphological and molecular characterisation of one new and several known species of the reniform nematode, *Rotylenchulus* Linford & Oliveira, 1940 (Hoplolaimidae: Rotylenchulinae), and a phylogeny of the genus. *Nematology* 18, 67–107. DOI: 10.1163/15685411-00002945

Vrain, T.C., Wakarchuk, D.A., Lévesque, A.C. and Hamilton, R.I. (1992) Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563–573.

Waeyenberge, L., Ryss, A., Moens, M., Pinochet, J. and Vrain, T.C. (2000) Molecular characterisation of 18 *Pratylenchus* species using rDNA restriction fragment length polymorphism. *Nematology* 2, 135–142. DOI: 10.1163/156854100509024

Waeyenberge, L., Viaene, N. and Moens, M. (2009) Species-specific duplex PCR for the detection of *Pratylenchus penetrans. Nematology* 11, 847–857. DOI: 10.1163/156854109X428016

Waite, I.S., O'Donnell, A.G., Harrison, A. *et al*. (2003) Design and evaluation of nematode 18S rDNA primers for PCR and denaturing gradient gel electrophoresis (DGGE) of soil community DNA. *Soil Biology and Biochemistry* 35, 1165–1173. DOI: 10.1016/S0038-0717(03)00177-9

Wang, H., Zhuo, K., Ye, W. and Liao, J.L. (2015) Morphological and molecular characterisation of *Pratylenchus parazeae* n. sp. (Nematoda: Pratylenchidae) parasitizing sugarcane in China. *European Journal of Plant Pathology* 143, 173–191. DOI: 10.1007/s10658-015-0674-z

Wang, X., Bosselut, N., Castagnone, C., Voisin, R., Abad, P. and Esmenjaud, D. (2003) Multiplex polymerase chain reaction identification of single individuals of the longidorid nematodes *Xiphinema index, X. diversicaudatum, X. vuittenezi,* and *X. italiae* using specific primers from ribosomal genes. *Phytopathology* 93, 160–166. DOI: 10.1094/PHYTO.2003.93.2.160

Yan, G.P., Smiley, R.W., Okubara, P.A., Skantar, A., Easley, S.A., Sheedy, J.G. and Thompson, A.L. (2008) Detection and discrimination of *Pratylenchus neglectus* and *P. thornei* in DNA extracts from soil. *Plant Disease* 92, 1480–1487. DOI: 10.1094/PDIS-92-11-1480

Yan, G.P., Smiley, R.W., Okubara, P.A., Skantar, A.M. and Reardon, C.L. (2013) Developing a real-time PCR assay for detection and quantification of *Pratylenchus neglectus* in soil. *Plant Disease* 97, 757–764. DOI: 10.1094/PDIS-08-12-0729-RE

Ye, W., Zeng, Y. and Kerns, J. (2015) Molecular characterization and diagnosis of root-knot nematodes (*Meloidogyne* spp.) from turfgrasses in North Carolina, USA. *PLoS One.* 10.1371/journal.pone.0143556: 1–16.

Yoder, M., Tandingan De Ley, I., King, I. *et al*. (2006) DESS: A versatile solution for preserving morphology and extractable DNA of nematodes. *Nematology* 8, 367–376. DOI:·10.1163/156854106778493448

Zheng, J., Subbotin, S.A., Waeyenberge, L. and Moens, M. (2000) Molecular characterisation of Chinese *Heterodera glycines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. *Russian Journal of Nematology* 8, 109–113.

Zijlstra, C. (2000) Identification of *Meloidogyne chitwoodi, M. fallax* and *M. hapla* based on SCAR-PCR: a powerful way of enabling reliable identification of populations or individuals that share common traits. *European Journal of Plant Pathology* 106, 283–290. DOI: 10.1023/A:1008765303364

Zijlstra, C., Donkers-Venne, D.T.H.M. and Fargette, M. (2000) Identification of *Meloidogyne incognita, M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. *Nematology* 2, 847–853. DOI: 10.1163/156854100750112798

Zijlstra, C., van Hoof, R. and Donkers-Venne, D. (2004) A PCR test to detect the cereal root-knot nematode *Meloidogyne naasi*. *European Journal of Plant Pathology* 110, 855–860. DOI: 10.1007/s10658-004-2492-6

13 Isolation and Characterization of Tandem Repeats in Nematode Genomes

PHILIPPE CASTAGNONE-SERENO

INRAE, Université Côte d'Azur, CNRS, ISA, Sophia Antipolis, France

13.1 Introduction

The highly repetitive fraction of the eukaryotic genome comprises sequences that are repeated in many copies, generally >10^3 copies. Such DNA sequences with high copy numbers that lie adjacent to each other in a block, or an array, are called tandem repeats. Tandem repeats are made of successive identical or nearly identical (degenerate) repeat units that usually vary in length from a few base pairs (bp) up to several hundred bp. According to the size of the repeat units, three arbitrary categories have been defined:

- The largest repeats (generally >70 bp) constitute **satellite DNA** (satDNA), which is abundant in constitutive heterochromatin and involved in chromosome structures such as centromeres and telomeres. SatDNA may constitute from less than 1% to up to 70% of the genome and exhibit huge variation in both sequence and copy number among species. Because of these peculiarities, satDNA has proven to be a relevant taxonomic marker in many organisms, including nematodes (Grenier *et al*., 1997).
- **Minisatellites** are shorter tandem repeats, which are enriched in subtelomeric regions of chromosomes. They are often highly polymorphic as to the number of repeat units in an array and can be used as genetic markers, the so-called variable number of tandem repeats (VNTR). Note that VNTRs are also common in mitochondrial DNA, particularly in nematodes (e.g. Whipple *et al*., 1998). However, because there has been little critical evaluation of the use of mitochondrial VNTR analysis as a practical genetic marker (Lunt *et al*., 1998), minisatellites will not be considered further here.
- **Microsatellites**, also known as simple sequence repeats (SSR), have been found in the genome of every eukaryotic organism analysed so far as repeat units, typically 1–6 bp, with repeat length rarely exceeding hundreds of repetitions in order. A very high mutation rate is usually associated with microsatellite loci, resulting in high heterozygosity and the presence of multiple alleles at a given locus. Since they are co-dominant, these markers have emerged as one the most popular and versatile neutral markers in a wide range of topics including, among others, genetic diversity, population structure and phylogeny.

This chapter will provide an overview of the practical methodologies that can be used to identify and characterize the tandem repeats that are most frequently used as genetic markers in nematodes, namely satellite DNA and microsatellites. However, the objective here is not to provide turnkey protocols (many variants have been

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
10.1079/9781786391759.0013

published in the specialized literature), but rather to return to the main principles that govern these protocols. Case studies on nematodes will serve to illustrate the point. In that respect, two well-defined situations are to be considered, depending on whether genomic resources for the species under investigation are available or not.

13.2 Bench Strategies for the Identification of Tandem Repeats

In the case where genomic resources are not available for the species of interest, wet-lab molecular biological techniques have to be used to identify tandem repeats.

13.2.1 The case of satellite DNA

Basically, the current strategy to detect a satellite DNA in a genome relies on the tandem repeat structure of this type of element, in particular the presence of restriction sites conserved between the repeating units (and their eventual loss due to mutations). Indeed, when it is digested with a restriction enzyme that releases the monomeric unit, the corresponding genomic DNA is fragmented predominantly into monomers, then into fragments corresponding to multimers when the restriction sites have disappeared (Fig. 13.1).

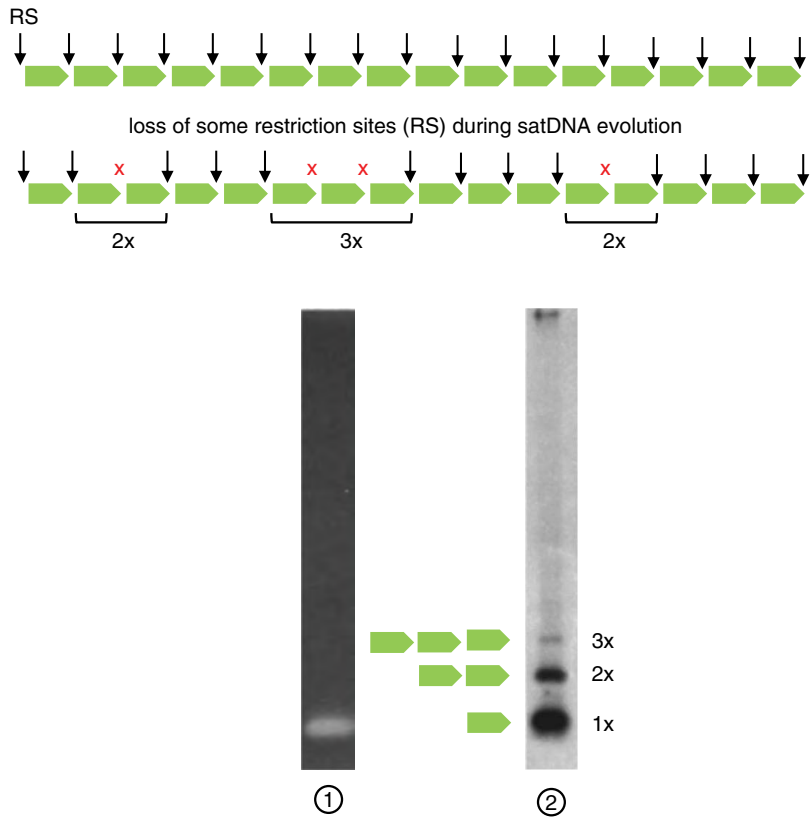

Fig. 13.1. The bench procedure for detection of satellite DNA by restriction analysis of genomic DNA. Satellite DNA (satDNA) is organized as long arrays of tandemly repeated elements, called monomers, containing a conserved restriction site (RS). During evolution, random mutations may affect the RS. Digestion of genomic DNA with the appropriate endonuclease will release monomers and multimers (here mono-, di- and trimers). (1) In practice, unless there is a very high genomic content of the satDNA, only the monomers can been visualized after digestion and ethidium bromide-stained gel electrophoresis. (2) Following further purification and cloning of the monomer and its use as a probe in a Southern blot experiment, a typical ladder pattern will reveal monomers and multimers.

Practically, the amount of satellite DNA in the genome of interest will determine the experimental modalities of its detection. If it represents more than 1% of the genome, it can be detected directly after digestion and agarose gel electrophoresis. In this case, the monomers form an intense band at the bottom of the gel, which is directly revealed by UV transillumination. In the opposite case, it is necessary to carry out a Southern blot of the electrophoresis gel resulting from the digestion, followed by the hybridization of the membrane thus obtained with the total genomic DNA that has been labelled previously (radioactively or at least once using a cold probe, according to standard methodologies). After revelation, the autoradiography will present the low band corresponding to the monomers.

At the end of this first step of detection (directly or after hybridization), the DNA fragments contained in the low band (multiple, individual copies of the monomeric unit) are cloned and used further as a molecular probe in a new Southern blot experiment (after dehybridization, the same membrane as above can be used). After revelation, the autoradiography will then reveal a typical ladder profile corresponding to the monomers and multimers, characteristic of the tandem organization of satellite DNA (Fig. 13.1).

In practical terms, restriction ranges are made beforehand to identify the enzyme(s) that will release the monomeric unit of repetition. For this, it is recommended to select endonucleases that have a four-base-pair restriction site (e.g. *Alu*I, *Hae*III, *Rsa*I, *Sau*3A, *Taq*I, etc.), because they cut DNA statistically more often than six-base-pair enzymes.

The examples below show the successful application of this methodology in plant-parasitic or entomopathogenic nematodes. These include, among others, root-knot nematodes, *Meloidogyne* spp. (Castagnone-Sereno *et al*., 1998; Randig *et al*., 2009) and the pinewood nematode, *Bursaphelenchu xylophilus* (Tarès *et al*., 1993), for the first category, and the entomopathogenic species, *Heterorhabditis indica* (Abadon *et al*., 1998) and *Steinernema carpocapsae* (Grenier *et al*., 1995), for the second.

13.2.2 The case of microsatellites

The original, and often still popular, method for the isolation of microsatellite markers consists in a three-step procedure: (i) enrichment of genomic DNA for microsatellite motifs cloning; (ii) screening of the resulting library; and (iii) sequencing of the positive clones. Briefly, high-quality genomic DNA from the target species is fragmented either using restriction enzymes or by sonication. Fragmented DNA is then size selected to obtain preferentially small fragments (in the range of 300–700 bp). Selected DNA fragments are then ligated into a common plasmid vector, and the ligation product used for the transformation of bacterial cells using classical molecular biology protocols. This transformation step generally yields thousands of recombinant clones that can be screened further for the effective presence of microsatellite sequences. Screening for positive clones is generally done by means of either Southern hybridization using repeat-containing probes, after blotting bacterial colonies onto nylon membranes, or polymerase chain reaction (PCR). Sequencing of the positive clones by the Sanger method (Green and Sambrook, 2014) is the final step to confirm definitely the isolation of microsatellite loci. Following the identification of clones containing a microsatellite, specific primers that frame the repeat motif are then designed and PCR conditions (i.e. reagent concentrations, thermal cycling conditions, etc.) are optimized (Lorenz, 2012) to allow the amplification of each locus from different individuals of a population (Fig. 13.2). For a thorough review of the various methods of microsatellite isolation, with their technical limits and drawbacks, refer to the article by Zane *et al*. (2002), which provides useful guidelines in making appropriate choices among the large panel of technical options currently available in the literature.

In nematodes of agronomic interest, the search for microsatellite markers was initiated in the early 2000s. Many technical variations of the traditional protocol have been used since then for their isolation, as for example in *Globodera* spp. (Thiéry and Mugniéry, 2000), *Heterodera schachtii* (Plantard and Porte, 2003), *Xiphinema index* (Villate *et al*., 2009), *Rotylenchulus reniformis* (Arias *et al*., 2009), *B. xylophilus* (Jung *et al*., 2010), *M. incognita* (Mulet *et al*., 2001) or in the entomopathogenic species *H. bacteriophora* (Bai *et al*., 2009).

However, although very common in laboratories, this general and most commonly used procedure is rather difficult to handle, time consuming and costly. Moreover, it generally uses a few specific repeated

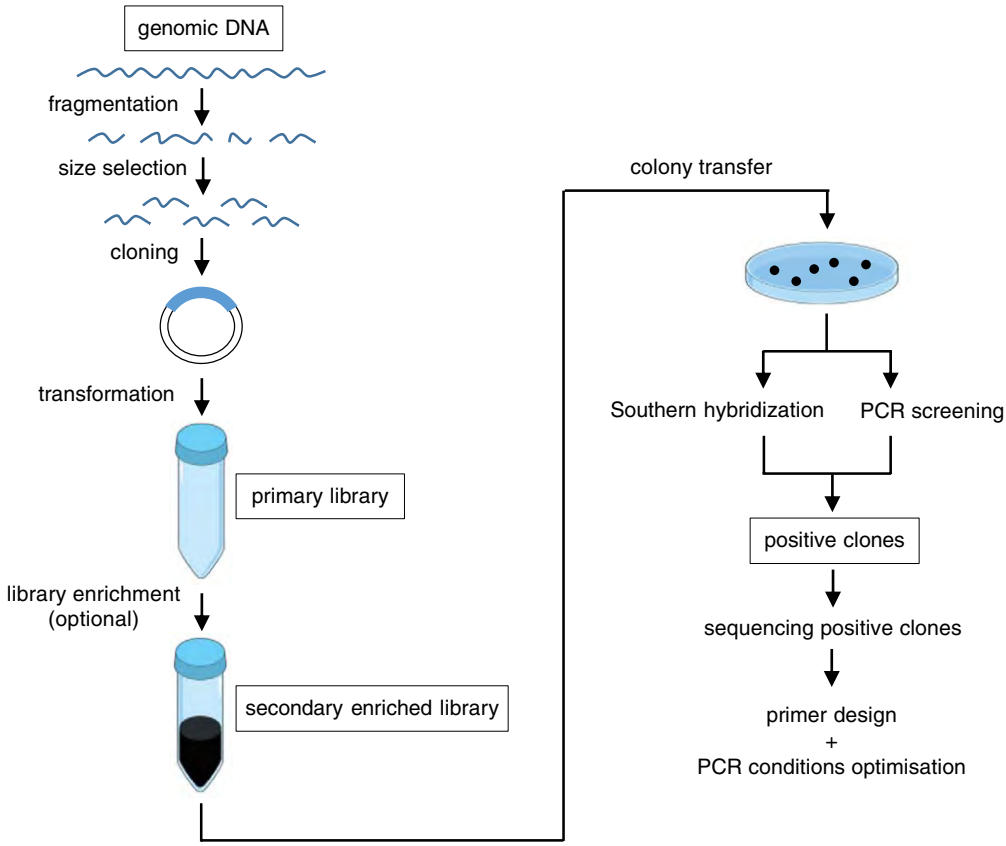

Fig. 13.2. Schematic representation of classical bench procedures for microsatellite isolation. The microsatellite enrichment step is optional but highly recommended. See Zane *et al*. (2002) for a detailed review of enrichment protocols.

motifs as probes, generally selected without prior knowledge of their abundance in the genome, thus introducing potential bias in genome representativeness. In addition, only a low number of suitable markers are generally obtained at the end of the process because of the high cost of Sanger sequencing.

In recent years, the development of high-throughput next-generation DNA sequencing (NGS) technologies has opened up new opportunities for microsatellite isolation in non-model organisms. As an alternative to traditional protocols, coupling library enrichment (see above) and NGS can speed up the acquisition of high-quality microsatellite loci at much lower cost, and hundreds or even thousands of microsatellites can usually be detected in a single experiment, allowing for stringent downstream selection of the most suitable loci for marker development (Santana *et al*., 2009; Weising *et al*., 2015). In particular, 454 pyrosequencing has become a sequencing platform of choice for developing microsatellites (Schoebel *et al*., 2013). For example, a high-throughput method based on coupling multiplex microsatellite enrichment and sequencing on a 454 GS-FLX Titanium platform allowed the direct isolation of 199 microsatellite loci for *B. xylophilus* (Malausa *et al*., 2011).

13.3 *In silico* Identification of Tandem Repeats

Within the past several years, both new sequencing technologies (e.g. NGS) and software resources have emerged, together with the growing availability of genomic datasets in the form of sequence reads, assembled or not. These new tools and resources have modified tremendously the strategies used for *de novo* identification of tandem repeats in genomes, as reviewed for satellite DNA (Lower *et al*., 2018) and microsatellites (Hodel *et al*., 2016).

Over the past decade, genomic data covering an increasing number of nematode species of agronomic interest have been deposited in public databases, and have became freely available to the scientific community (e.g. http://www.nematode.net/NN3_frontpage.cgi; https://parasite.wormbase.org/index.html). In this situation, the most parsimonious approach for tandem repeat discovery (either microsatellites or satellite DNA) is to mine the corresponding resource with appropriate software. Table 13.1 presents a non-comprehensive list of software packages used for assessing tandem repeats from NGS data. Interestingly, some of these software packages allow the detection of either microsatellite loci or satellite DNA repeat units, according to their initial parameterization (e.g. Phobos). In addition, from a practical point of view, many of these software packages interact with Primer3 (Untergasser *et al*., 2012), a very popular and widely used program for designing PCR primers.

However, when working with non-model organisms, it is often necessary to generate sequence data before computational analysis as indicated above. In this case, the choice of the NGS sequencing platform is of particular importance. Read length greatly affects the ability to discover microsatellite markers, since longer reads will enhance the possibility to include the flanking regions needed for primer design. In that respect, the Roche 454 platform has been used preferentially for microsatellite development compared to the Illumina platform (Castoe *et al*., 2012). This aspect is even more important for the detection of monomeric sequences of satellite DNA and the possibilities offered by new technologies such as PacBio, Oxford Nanopore, etc. to obtain significantly longer reads seem very promising.

In the specific case of microsatellites, it should be noted that the limiting factor for the development of markers that can be used in population genetics is no longer loci identification (either from pre-existing or *de novo* generated sequence data), but their subsequent validation as genotyping tools, since thousands of loci are generally retrieved (Wei *et al*., 2014). For example, in *M. incognita*, mining the nematode genome with msatfinder led to the identification of 2183 microsatellites (excluding mono-nucleotide repeats) that appeared suitable to design markers for population genetic studies (Castagnone-Sereno *et al*., 2010). Similarly, in the plant-parasitic nematode, *Subanguina moxae*, MISA analysis of the genome assembly of Illumina pair-end reads produced a final set of 2243 simple sequence microsatellite loci (Takeuchi *et al*., 2015).

Table 13.1. Some software packages used for assessing tandem repeats from NGS data. This table is not comprehensive.

Software	URL	Reference
For microsatellites		
MISA	http://misaweb.ipk-gatersleben.de/	Beier *et al*. (2017)
msatcommander	http://code.google.com/p/msatcommander/	Faircloth (2008)
msatfinder	http://web.archive.org/web/20071026090642/http://www.genomics.ceh.ac.uk/msatfinder/	Thurston and Field (2005)
PAL_FINDER	http://sourceforge.net/projects/palfinder/	Castoe *et al*. (2012)
Phobos	http://www.rub.de/ecoevo/cm/cm_phobos.htm	Mayer (2010)
QDD	http://net.imbe.fr/~emeglecz/qdd.html	Meglécz *et al*. (2014)
For satellite DNA		
Phobos	http://www.rub.de/ecoevo/cm/cm_phobos.htm	Mayer (2010)
RepeatExplorer	http://www.repeatexplorer.org	Novák *et al*. (2013)
Tandem Repeats Finder	https://tandem.bu.edu/trf/trf.html	Benson (1999)
TAREAN	http://w3lamc.umbr.cas.cz/lamc/resources.php	Novák *et al*. (2017)

13.4 The Contribution of Tandem Repeats as Molecular Markers in Nematodes

13.4.1 Satellite DNA-based species-specific diagnostic tools

Satellite DNAs are located in heterochromatic chromosomal regions and are involved in processes related to complex structural and functional features of eukaryotic chromosomes, particularly in centromeric and pericentromeric regions (Csink and Henikoff, 1998). Satellite DNA repeats evolve in a concerted manner, being continuously homogenized within a genome and fixed within a group of reproductively linked organisms (Dover, 2002). Notably, dynamics of satellite DNA content and composition can affect genome functions and evolution, and may result in reproductive isolation and speciation (Plohl, 2010). Indeed, satellite DNA families can be unique to, or extremely abundant in, a given species and at an undetectable level in another, even closely related species. When experimentally demonstrated, the species specificity of such genomic sequences constitutes a feature of great potential for the development of diagnostic methodologies. In the case of nematodes of agronomic interest, satellite DNA sequences have been mostly developed as molecular tools for the specific detection of a range of species, using either hybridization or PCR-based protocols. Some typical examples are illustrated in Table 13.2.

13.4.2 Microsatellites and population genetic studies in plant-parasitic nematodes

Microsatellite analysis remains one of the most popular genotyping methods because of its low cost of development and ease of analysis. These codominant markers are distributed throughout the genome, and the high frequency at which mutations occur at these sites results in an increase or decrease in the number of elements in the array, which produces the high level of polymorphism required for population genetic analysis (Ellegren, 2004).

Owing to these properties, microsatellites have been widely used to make inferences on several aspects of population genetics in plant-parasitic nematodes. These markers proved to be powerful tools to describe the genetic diversity and population structure of a wide range of species, including several cyst nematode species from the genera *Heterodera* and *Globodera* (Plantard and Porte, 2004; Montarry *et al*., 2015; Wang *et al*., 2015; Gracianne *et al*., 2016) or the pinewood nematode, *B. xylophilus* (Mallez *et al*., 2013). They also allowed discovery of signs of cryptic sex in the genome of mainly asexual species, e.g. the identification of rare sexual reproduction events in *X. index* (Villate *et al*., 2010). Finally, microsatellites also contributed to the deciphering of the origin and phylogeography of some important species, such as the potato cyst nematodes (Plantard *et al*., 2008; Boucher *et al*., 2013) and the pinewood nematode (Jung *et al*., 2010; Mallez *et al*., 2015).

Table 13.2. Some examples of the use of satellite DNA as a species-specific molecular marker for the purpose of diagnostics in plant-parasitic or entomopathogenic nematodes.

Species	Practical approach	Reference
Entomopathogenic nematodes		
Heterorhabditis indica	Hybridization (slot-blot)	Stack *et al*. (2000)
Steinernema carpocapsae	Hybridization (squash-blot)	Simard *et al*. (2007)
Steinernema feltiae	Hybridization (squash-blot)	Simard *et al*. (2007)
Steinernema glaseri	Hybridization (squash-blot)	Simard *et al*. (2007)
Plant-parasitic nematodes		
Bursaphelenchus xylophilus	Conventional PCR	Cardoso *et al*. (2012)
Bursaphelenchus xylophilus	Real-time PCR	François *et al*. (2007)
Meloidogyne exigua	Hybridization (squash-blot)	Randig *et al*. (2002)
Meloidogyne hapla	Conventional PCR	Castagnone-Sereno *et al*. (1995)
Meloidogyne hapla	Hybridization (squash-blot)	Piotte *et al*. (1995)
Pratylenchus thornei	Hybridization (dot-blot)	Carrasco-Ballesteros *et al*. (2007)

13.5 Concluding Remarks

The advent of ever more efficient and resolving sequencing technologies and bioinformatic resources has upset the way genomes are studied, including when the identification of tandem repeats is concerned. In the case of non-model organisms, such as most nematodes of agronomic interest, NGS now makes it easy to obtain genomic resources at constantly decreasing costs. This technological shift has changed the focus of research aimed at developing genetic markers, the limiting factor no longer being the identification of potentially interesting genomic regions, but their subsequent validation as practical tools. However, from this point of view, the manipulation step 'at the bench' is still essential.

In recent years, single-nucleotide polymorphisms (SNPs) have started to compete with microsatellites as genotyping markers in population genetic studies (Guichoux *et al*., 2011), although this trend has been poorly followed until now in nematode research. At the same time, the consistency and robustness of the information provided by these two types of markers have been questioned, with contrasted outputs (e.g. Fischer *et al*., 2017; Tsykun *et al*., 2017). However, because of their great applicability (high polymorphism, easy scoring, cost-efficiency), it has been argued, with relevance, that microsatellites will remain in the near future neutral molecular markers of choice in a number of case studies (Hodel *et al*., 2016).

13.6 References

Abadon, M., Grenier, E., Laumond, C. and Abad, P. (1998) A species-specific satellite DNA from the entomopathogenic nematode *Heterorhabditis indicus*. *Genome* 41, 148–153. DOI: 10.1139/g98-005

Arias, R.S., Stetina, S.R., Tonos, J.L., Scheffler, J.A. and Scheffler, B.E. (2009) Microsatellites reveal genetic diversity in *Rotylenchulus reniformis* populations. *Journal of Nematology* 41, 146–156

Bai, X., Saeb, A.T.M., Andrew, M. and Grewal, P.S. (2009) Isolation and characterization of microsatellite loci in the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Molecular Ecology Resources* 9, 207–209. DOI: 10.1111/j.1755-0998.2008.02369.x

Beier, S., Thiel, T., Münch, T., Scholz, U. and Mascher, M. (2017) MISA-web: a web server for microsatellite prediction. *Bioinformatics* 33, 2583–2585. DOI: 10.1093/bioinformatics/btx198

Benson, G. (1999) Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Research* 27, 573–580. DOI : 10.1093/nar/27.2.573

Boucher, A.C., Mimee, B., Montarry, J., Bardou-Valette, S., Bélair, G., Moffett, P. and Grenier, E. (2013) Genetic diversity of the golden potato cyst nematode *Globodera rostochiensis* and determination of the origin of populations in Quebec, Canada. *Molecular Phylogenetics and Evolution* 69, 75–82. DOI: 10.1016/j.ympev.2013.05.020

Cardoso, J.M.S., Fonseca, L. and Abrantes, I. (2012) Direct molecular detection of the pinewood nematode, *Bursaphelenchus xylophilus*, from pine wood, bark and insect vector. *European Journal of Plant Pathology* 133, 419–425. DOI: 10.1007/s10658-011-9915-y

Carrasco-Ballesteros, S., Castillo, P., Adams, B.J. and Pérez-Artés, E. (2007) Identification of *Pratylenchus thornei*, the cereal and legume root-lesion nematode, based on SCAR-PCR and satellite DNA. *European Journal of Plant Pathology* 118, 115–125. DOI: 10.1007/s10658-007-9110-3

Castagnone-Sereno, P., Esparrago, G., Abad, P., Leroy, F. and Bongiovanni, M. (1995) Satellite DNA as a target for PCR specific detection of the plant-parasitic nematode *Meloidogyne hapla*. *Current Genetics* 28, 566–570. DOI: 10.1007/BF00518170

Castagnone-Sereno, P., Semblat, J.P., Leroy, F. and Abad, P. (1998) A new *Alu* I satellite DNA in the root-knot nematode *Meloidogyne fallax*: Relationships with satellites from the sympatric species *M. hapla* and *M. chitwoodi*. *Molecular Biology and Evolution* 15, 1115–1122. DOI: 10.1093/oxfordjournals.molbev.a026019

Castagnone-Sereno, P., Danchin, E.G.J., Deleury, E., Guillemaud, T., Malausa, T. and Abad, P. (2010) Genome-wide survey and analysis of microsatellites in nematodes, with a focus on the plant-parasitic species *Meloidogyne incognita*. *BMC Genomics* 11, 598. DOI: 10.1186/1471-2164-11-598

Castoe, T.A., Poole, A.W., de Koning, A.P.J. *et al*. (2012) Rapid microsatellite identification from Illumina paired-end genomic sequencing in two birds and a snake. *PLoS One* 7, e30953. DOI: 10.1371/journal.pone.0030953

Csink, A.K. and Henikoff, S. (1998) Something from nothing: the evolution and utility of satellite repeats. *Trends in Genetics* 14, 200–204. DOI: 10.1016/S0168-9525(98)01444-9

Dover, G.A. (2002) Molecular drive. *Trends in Genetics* 18, 587–589. DOI: 10.1016/S0168-9525(02)02789-0

Ellegren, H. (2004) Microsatellites: simple sequences with complex evolution. *Nature Reviews Genetics* 5, 435–445. DOI: 10.1038/nrg1348

Faircloth, B.C. (2008) MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources* 8, 92–94. DOI: 10.1111/j.1471-8286.2007.01884.x

Fischer, M.C., Rellstab, C., Leuzinger, M., Roumet, M., Gugerli, F., Shimizu, K.K., Holderegger, R. and Widmer, A. (2017) Estimating genomic diversity and population differentiation - an empirical comparison of microsatellite and SNP variation in *Arabidopsis halleri. BMC Genomics* 18,69. DOI: 10.1186/s12864-016-3459-7

François, C., Castagnone, C., Boonham, N. *et al*. (2007) Satellite DNA as a target for TaqMan real-time PCR detection of the pinewood nematode, *Bursaphelenchus xylophilus. Molecular Plant Pathology* 8, 803–809. DOI: 10.1111/J.1364-3703.2007.00434.X

Guichoux, E., Lagache, L., Wagner, S. *et al*. (2011) Current trends in microsatellite genotyping. *Molecular Ecology Resources* 11, 591–611. DOI: 10.1111/J.1755-0998.2011.03014.X

Gracianne, C., Jan, P.L., Fournet, S., Olivier, E., Arnaud, J.F., Porte, C., Bardou-Valette, S., Denis, M.C. and Petit, E.J. (2016) Temporal sampling helps unravel the genetic structure of naturally occurring populations of a phytoparasitic nematode. 2. Separating the relative effects of gene flow and genetic drift. *Evolutionary Applications* 9, 1005–1016. DOI: 10.1111/eva.12401

Green, M.R. and Sambrook, J. (2014) *Molecular Cloning: A Laboratory Manual*, 4th edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, New York, USA.

Grenier, E., Laumond, C. and Abad, P. (1995) Characterization of a species-specific satellite DNA from the entomopathogenic nematode *Steinernema carpocapsae*. *Molecular and Biochemical Parasitology* 69, 93–100. DOI: 10.1016/0166-6851(94)00197-U

Grenier, E., Castagnone-Sereno, P. and Abad, P. (1997) Satellite DNA sequences as taxonomic markers in nematodes of agronomic interest. *Parasitology Today* 13, 398–401. DOI: 10.1016/S0169-4758(97)01113-7

Hodel, R.G.J., Segovia-Salcedo, M.C., Landis, J.B. *et al*. (2016) The report of my death was an exaggeration: A review for researchers using microsatellites in the 21st century. *Applications in Plant Sciences* 4, e1600025. DOI: 10.3732/apps.1600025

Jung, J., Han, H., Ryu, S.H. and Kim, W. (2010) Microsatellite variation in the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle in South Korea. *Genes & Genomics* 32, 151–158. DOI: 10.5423/PPJ.NT.09.2012.0135

Lower, S.S., McGurk, M.P., Clark, A.G. and Barbash, D.A. (2018) Satellite DNA evolution: old ideas, new approaches. *Current Opinion in Genetics and Development* 49, 70–78. DOI: 10.1016/j.gde.2018.03.003

Lorenz, T.C. (2012) Polymerase Chain Reaction: Basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments* 63, e3998. DOI: 10.3791/3998

Lunt, D.H., Whipple, L.E. and Hyman, B.C. (1998) Mitochondrial DNA variable number tandem repeats (VNTRs): utility and problems in molecular ecology. *Molecular Ecology* 7, 1441–1455. DOI: 10.1046/j.1365-294x.1998.00495.x

Malausa, T., Gilles, A., Meglécz, E. *et al*. (2011) High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. *Molecular Ecology Resources* 11, 638–644. DOI: 10.1111/J.1755-0998.2011.02992.X

Mallez, S., Castagnone, C., Espada, M., Vieira, P., Eisenback, J.D., Mota, M., Guillemaud, T. and Castagnone-Sereno P. (2013) First insights into the genetic diversity of the pinewood nematode in its native area using new polymorphic microsatellite loci. *PLoS One* 8, e59165. DOI: 10.1371/journal.pone.0059165

Mallez, S., Castagnone, C., Espada M. *et al*. (2015) Worldwide invasion routes of the pinewood nematode: What can we infer from population genetics analyses? *Biological Invasions* 17, 1199–1213. DOI: 10.1007/s10530-014-0788-9

Mayer, C. (2010) Phobos - a tandem repeat search tool for complete genomes - Version 3.3.12. Available at: http://www.rub.de/ecoevo/cm/cm_phobos.htm (accessed 22 July 2020).

Meglécz, E., Pech, N., Gilles, A., Dubut, V., Hingamp, P., Trilles, A., Grenier, R. and Martin, J.F. (2014) QDD version 3.1: A user friendly computer program for microsatellite selection and primer design revisited: experimental validation of variables determining genotyping success rate. *Molecular Ecology Resources* 14, 1302–1313. DOI: 10.1111/1755-0998.12271

Montarry, J., Jan, P.L., Gracianne, C., Overall, A.D.J., Bardou-Valette, S., Olivier, E., Fournet, S., Grenier, E. and Petit, E.J. (2015) Heterozygote deficits in cyst plant-parasitic nematodes: possible causes and consequences. *Molecular Ecology* 24, 1654–1677. DOI: 10.1111/mec.13142

Mulet, K., Fargette, M., Richaud, M., Genson, G. and Castagnone-Sereno, P. (2001) Isolation of microsatellites from an enriched genomic library of the plant-parasitic nematode *Meloidogyne incognita* and their detection in other root-knot nematode species. *European Journal of Plant Pathology* 129, 501–505. DOI: 10.1007/s10658-010-9721-y

Novák, P., Neumann, P., Pech, J., Steinhaisl, J. and Macas, J. (2013) RepeatExplorer: a Galaxy-based web server for genome-wide characterization of eukaryotic repetitive elements from next-generation sequence reads. *Bioinformatics* 29, 792–793. DOI: 10.1093/bioinformatics/btt054

Novák, P., Robledillo, L.A., Koblížková, A., Vrbová, I., Neumann, P. and Macas, J. (2017) TAREAN: a computational tool for identification and characterization of satellite DNA from unassembled short reads. *Nucleic Acids Research* 45, e111. DOI: 10.1093/nar/gkx257

Piotte, C., Castagnone-Sereno, P., Bongiovanni, M., Dalmasso, A. and Abad, P. (1995) Analysis of a satellite DNA from *Meloidogyne hapla* and its use as a diagnostic probe. *Phytopathology* 85, 458–462. DOI: 10.1094/Phyto-85-458

Plantard, O. and Porte, C. (2003) Isolation and characterization of microsatellite loci in the sugar beet cyst nematode *Heterodera schachtii. Molecular Ecology Notes* 3, 139–141. DOI: 10.1046/j.1471-8286.2003.00383.x

Plantard, O. and Porte, C. (2004) Population genetic structure of the sugar beet cyst nematode *Heterodera schachtii*: a gonochoristic and amphimictic species with highly inbred but weakly differentiated populations. *Molecular Ecology* 13, 33–41. DOI: 10.1046/j.1365-294X.2003.02023.x

Plantard, O., Picard, D., Valette, S., Scurrah, M., Grenier, E. and Mugniéry, D. (2008) Origin and genetic diversity of Western European populations of the potato cyst nematode (*Globodera pallida*) inferred from mitochondrial sequences and microsatellite loci. *Molecular Ecology* 17, 2208–2218. DOI: 10.1111/j.1365-294X.2008.03718.x

Plohl, M. (2010) Those mysterious sequences of satellite DNAs. *Periodicum Biologorum* 112, 403–410.

Randig, O., Bongiovanni, M., Carneiro, R.M.G., Sarah, J.-L. and Castagnone-Sereno, P. (2002) A species-specific satellite DNA family in the genome of the coffee root-knot nematode *Meloidogyne exigua*: application to molecular diagnostics of the parasite. *Molecular Plant Pathology* 3, 431–437. DOI: 10.1046/j.1364-3703.2002.00134.x

Randig, O., Deau, F., dos Santos, M.F.A., Tigano, M.S., Carneiro, R.M.D.G. and Castagnone-Sereno, P. (2009) A novel species-specific satellite DNA family in the invasive root-knot nematode *Meloidogyne mayaguensis* and its potential use for diagnostics. *European Journal of Plant Pathology* 125, 485–495. DOI: 10.1007/s10658-009-9497-0

Santana, Q.C., Coetzee, M.P.A., Steenkamp, E.T., Mlonyeni, O.X., Hammond, G.N.A., Wingfield, M.J. and Wingfield, B.D. (2009) Microsatellite discovery by deep sequencing of enriched genomic libraries. *BioTechniques* 46, 217–223. DOI: 10.2144/000113085

Schoebel, C.N., Brodbeck, S., Buehler, D. *et al*. (2013) Lessons learned from microsatellite development for nonmodel organisms using 454 pyrosequencing. *Journal of Evolutionary Biology* 26, 600–611. DOI: 10.1111/JEB.12077

Simard, L., Bélair, G., Stock, S.P., Mauleon, H. and Dionne, J. (2007) Natural occurrence of entomopathogenic nematodes (Rhabditida: Steinernematidae) on golf courses in eastern Canada. *Nematology* 9, 325–332. DOI: 10.1163/156854107781352070

Stack, C.M., Easwaramoorthy, S.G., Metha, U.K., Downes, M.J., Griffin, C.T. and Burnell, A.M. (2000) Molecular characterisation of *Heterorhabditis indica* isolates from India, Kenya, Indonesia and Cuba. *Nematology* 2, 477–487. DOI: 10.1163/156854100509321

Takeuchi, T., Yamaguchi, M., Tanaka, R., Dayi, M., Ogura, N. and Kikuchi, T. (2015) Development and validation of SSR markers for the plant-parasitic nematode *Subanguina moxae* using genome assembly of Illumina pair-end reads. *Nematology* 17, 515–522. DOI: 10.1163/15685411-00002885

Tarès, S., Lemontey, J.M., De Guiran, G. and Abad, P. (1993) Cloning and characterization of a highly conserved satellite DNA sequence specific for the phytoparasitic nematode *Bursaphelenchus xylophilus. Gene* 129, 269–273. DOI: 10.1016/0378-1119(93)90278-b

Thiéry, M. and Mugniéry, D. (2000) Microsatellite loci in the phytoparasitic nematode *Globodera. Genome* 43, 160–165. DOI: 10.1139/g99-106

Thurston, M.I. and Field, D. (2005) Msatfinder: detection and characterisation of microsatellites. Available at: http://web.archive.org/web/20071026090642/http://www.genomics.ceh.ac.uk/msatfinder/ (accessed 21 July 2020).

Tsykun, T., Rellstab, C., Dutech, C., Sipos, G. and Prospero, S. (2017) Comparative assessment of SSR and SNP markers for inferring the population genetic structure of the common fungus *Armillaria cepistipes. Heredity* 119, 371–380. DOI: 10.1038/hdy.2017.48

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M. and Rozen, S.G. (2012) Primer3-new capabilities and interfaces. *Nucleic Acids Research* 40, e115. DOI: 10.1093/nar/gks596

Villate, L., Esmenjaud, D., Coedel, S. and Plantard, O. (2009) Development of nine polymorphic microsatellite markers for the phytoparasitic nematode *Xiphinema index*, the vector of the grapevine fanleaf virus. *Molecular Ecology* 9, 229–232. DOI: 10.1111/j.1755-0998.2008.02411.x

Villate, L., Esmenjaud, D., Van Helden, M., Stoeckel, S. and Plantard, O. (2010) Genetic signature of amphimixis allows for the detection and fine scale localization of sexual reproduction events in a mainly parthenogenetic nematode. *Molecular Ecology* 19, 856–873. DOI: 10.1111/j.1365-294X.2009.04511.x

Wang, H.M., Zhao, H.H. and Chu, D. (2015) Genetic structure analysis of populations of the soybean cyst nematode, *Heterodera glycines*, from north China. *Nematology* 17, 591–600. DOI: 10.1163/15685411-00002893

Wei, N.A., Bemmels, J.B. and Dick, C.W. (2014) The effects of read length, quality and quantity on microsatelliote discovery and primer development: from Illumina to PacBio. *Molecular Ecology Resources* 14, 953–-965. DOI: 10.1111/1755-0998.12245

Weising, K., Woehrmann, T. and Huettel, B. (2015) The use of high-throughput DNA sequencing for microsatellite discovery in plants. In: Hörandl, E. and Appelhans, M.S. (eds) *Next-generation Sequencing in Plant Systematics*. Koeltz Scientific Books, Königstein, Germany, pp.205–269

Whipple, L.E., Lunt, D.H. and Hyman, B.C. (1998) Mitochondrial DNA length variation in *Meloidogyne incognita* isolates of established genetic relationships: utility for nematode population studies. *Fundamental and Applied Nematology* 21, 265–271.

Zane, L., Bargelloni, L. and Patarnello, T. (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology* 11, 1–16. DOI: 10.1046/j.0962-1083.2001.01418

14 Characterization of Nematode Mitochondrial Genomes

DANNY A. HUMPHREYS-PEREIRA,[1] TAEHO KIM[2] AND JOONG-KI PARK[2]

[1]*Laboratory of Nematology, University of Costa Rica, San José, Costa Rica;*
[2]*Division of EcoScience, Ewha Womans University, Seoul, Republic of Korea*

14.1 Introduction

Mitochondrial (mt) genomes are a useful source of molecular markers for species identification (Powers and Harris, 1993; Powers *et al.*, 2005; Palomares-Rius *et al.*, 2017a) and systematics and phylogenetic relationships among diverse nematode groups (Sultana *et al.*, 2013a; Humphreys-Pereira and Elling, 2014, 2015; Kim *et al.*, 2017; Palomares-Rius *et al.*, 2017b). In addition, partial mt DNA fragments have been used as a powerful marker for DNA barcoding, population genetic and phylogeographical studies (Plantard *et al.*, 2008; Gutiérrez-Gutiérrez *et al.*, 2011; Barrantes-Infante *et al.*, 2018; Subbotin *et al.*, 2018).

Most nematodes have a circular mt genome (Fig. 14.1) with 12 protein coding genes (PCGs: *cox1–3*, *nad1–6*, *nad4L*, *cob* and *atp6*), two ribosomal genes (rRNAs: *rrnS* and *rrnL*) and 22 transfer RNA genes (tRNAs). Contrary to the majority of metazoans, nematode mt genomes do not have the *atp8* gene, except for *Trichinella* and *Trichuris* (Lavrov and Brown, 2001; Liu *et al.*, 2012a,b). Furthermore, some nematode mt-tRNAs do not have the D-arm or the T-arm (Wolstenholme *et al.*, 1987; Okimoto and Wolstenholme, 1990; Watanabe *et al.*, 1994) and in a few cases, the mt-tRNAs lack both arms (Jühling *et al.*, 2012a, 2018).

The first two nematode mt genomes sequenced and annotated were from the animal-parasitic nematode, *Ascaris suum*, and the free-living nematode, *Caenorhabditis elegans* (Okimoto *et al.*, 1992). Currently, there are more than 300 complete nematode mt genome sequences available in GenBank (accessed October 2020). *Caenorhabditis* is the genus with more species mt genomes sequenced (15 species), followed by *Trichinella* and *Meloidogyne* with twelve and eight taxa, respectively (https://www.ncbi.nlm.nih.gov/genbank/).

More than twenty plant-parasitic nematode mt genomes have been sequenced and published so far: *Aphelenchoides besseyi*, *Bursaphelenchus xylophilus*, *B. mucronatus*, *Globodera ellingtonae*, *G. pallida*, *G. rostochiensis*, *Heterodera glycines*, *Longidorus vineacola*, *Meloidogyne arenaria*, *M. chitwoodi*, *M. enterolobii*, *M. floridensis*, *M. graminicola*, *M. hapla*, *M. incognita*, *M. javanica*, *Paralongidorus litoralis*, *Pratylenchus vulnus*, *Radopholus similis*, *Xiphinema americanum*, *X. pachtaicum*, *X. rivesi* and others (Armstrong *et al.*, 2000; He *et al.*, 2005; Gibson *et al.*, 2007, 2011; Jacob *et al.*, 2009; Sultana *et al.*, 2013a,b; Sun *et al.*, 2014a; Humphreys-Pereira and Elling, 2014, 2015; Besnard *et al.*, 2014; García and Sánchez-Puerta, 2015; Phillips *et al.*, 2016; Palomares-Rius *et al.*, 2017b). The mitochondrial genome of most of these is composed of a circular, single chromosome, but the mtDNA of *Globodera* spp. are multipartite (i.e. the mt

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
10.1079/9781786391759.0014

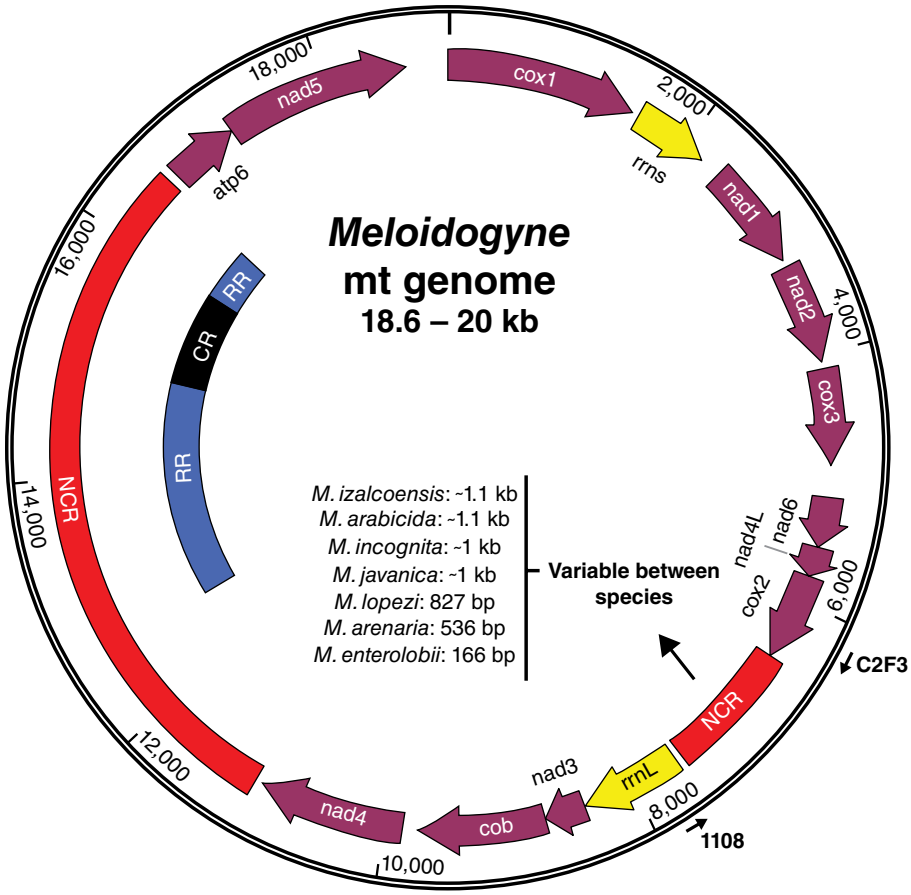

Fig. 14.1. Circular mitochondrial genome of *Meloidogyne* spp. *rrnL*, *16S* ribosomal RNA; *rrnS*, *12S* ribosomal RNA; *atp6*, ATP synthase subunit 6; *cob*, cytochrome b; *cox1–3*, cytochrome c oxidase subunits 1–3; *nad1–6*, and *nad4L*, nicotinamide adenine dinucleotide dehydrogenase subunits 1–6, and 4L; RR, tandem repeat region; CR, putative control region. The mt genome was illustrated with SnapGene®.

genome is divided into two or multiple chromosomes): the *G. ellingtonae* mt genome has two large circles, whereas the mt genomes of *G. pallida* and *G. rostochiensis* have at least five circles (Armstrong *et al.*, 2000; Gibson *et al.*, 2007; Phillips *et al.*, 2016). Among other unusual features, the PCGs in the enoplean mt genomes are transcribed from both strands (He *et al.*, 2005; Palomares-Rius *et al.*, 2017b). Also, in the burrowing nematode, *R. similis*, the amino acid tyrosine (Tyr) is encoded by the codon UAA, which normally encodes for a termination site in the nematode mt genomes (Jacob *et al.*, 2009).

Plant-parasitic nematode mt genomes are commonly obtained by using several approaches, i.e. PCR amplification and sequencing of two (Sultana *et al.*, 2013a) or several overlapped DNA fragments (Humphreys-Pereira and Elling, 2014, 2015). The amplified PCR fragments are cloned into a plasmid vector and/or directly sequenced using the primer walking method. Also, long PCR fragments can be sequenced with new technologies, such as next-generation sequencing (Margulies *et al.*, 2005; Shendure and Ji, 2008; Balasubramanian, 2015). These technologies have been performed successfully for sequencing the mt genomes of the plant-parasitic nematodes *G. ellingtonae*, *M. graminicola* and members of the family Longidoridae (Besnard *et al.*, 2014; Phillips *et al.*, 2016; Palomares-Rius *et al.*, 2017b).

14.2 PCR Amplification of Mitochondrial Genomes

Genomic DNA can be isolated with the phenol-chloroform extraction method or with a commercial DNA extraction kit from a pool of individuals or single nematodes. Also, mt DNA can be amplified from crude DNA extracts from juveniles or adults (see Subbotin, Chapter 12, this volume, for DNA extraction protocols).

Initially, partial gene fragments of the mt genome are amplified with degenerate, universal, or genera/species-specific primers already published (Table 14.1). Species-specific long-PCR primers are designed from the nucleotide sequences obtained from the partial gene fragments. Specific primers can be also designed from expressed sequence tags (ESTs) available in GenBank. ESTs can be blasted against the mt genomes of other plant-parasitic nematodes to search for mt protein-coding genes that can be used to design primers.

Long-range PCR can also be performed using nematode mt specific primers designed from previously published mt markers, e.g. in *Meloidogyne*, the DNA markers *cox1*, *cox2*, the region between the *cox2-16S* rRNA and *nad5* have been sequenced in several species (Powers and Harris, 1993; Humphreys-Pereira *et al.*, 2014; Kiewnick *et al.*, 2014; García and Sánchez-Puerta, 2015; Palomares-Rius *et al.*, 2017b). New universal or degenerate primers can be designed by aligning sequences from the chromadorean or enoplean mt genomes available in the GenBank or using primers already published.

The long-range PCR should be achieved with a high-fidelity *Taq* polymerase suitable for the amplification of mt DNA as it normally contains non-coding regions with high A+T-rich and tandem repeat sequence blocks. Caution must be taken when large DNA fragments are amplified because of the presence of poly(T) regions in the mitochondrial genomes. Some fragments that are ambiguous in their sequence can be amplified with a proofreading DNA polymerase to reduce the error rate (e.g. to avoid the insertion of extra thymine residues or artificial mutations).

14.3 Protocols for PCR

14.3.1 Amplification of partial mt genes/short DNA fragments (< 3 kb)

Partial digestion using PCR to obtain fragments is detailed in Subbotin, Chapter 12, this volume.

14.3.2 Long-range PCR

Once partial fragments are obtained from PCR and sequenced, they can be used to design species-specific primer sets for long-range PCR amplification.

Protocol 1: Long-range PCR with KOD Xtreme™ Hot Start DNA polymerase (Merck) (Humphreys-Pereira and Elling, 2014, 2015)

- Add 1–2 µl of crude DNA extract from a single individual or 1 µl of bulk DNA (50 ng μl^{-1}) to 0.2 ml PCR tube containing 12.5 µl 2× Xtreme Buffer, 5 µl dNTPs mixture (2 mM each), 0.75 µl of each primer (10 µM), 0.5 µl KOD Xtreme™ Hot Start DNA polymerase (1 U μl^{-1}) and nuclease-free water to a final volume of 25 µl.
- Transfer the 0.2 ml PCR tubes to a thermocycler and start the following PCR conditions: an initial denaturation at 94°C for 2 min; followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 50–66°C (depending on the primer) for 30 s, and extension at 68°C for 2–10 min (depending on the DNA fragment size, 1 min kb^{-1}). A final extension step is performed at 68°C for 10 min.

Protocol 2: Long-range PCR with *TaKaRa LA Taq* DNA polymerase (Takara Bio) (Kim *et al.*, 2014, 2018)

- Add 2 µl of template DNA to a 0.2 ml PCR tube containing 2.5 U *Taq* polymerase, 0.4 mM dNTP mixture, 1× LA Taq buffer, 2.5 mM $MgCl_2$, 10 pmol of each primer, and enough nuclease-free water to make a final total volume of 50 µl.
- Transfer the 0.2 ml PCR tubes to a thermocycler and set the following PCR conditions: an initial denaturation at 94–95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing and extension at 55–70°C (depending on the primer) for 2–15 min (depending on the DNA fragment size, 1 min kb^{-1}), and a final extension step at 68°C for 10 min.

Table 14.1. Primer sequences for the amplification of partial gene fragments from the mitochondrial genomes of plant-parasitic nematodes.

Primer combination[a]	Primer sequence (5′–3′)	Primer reference	DNA region	Target nematode	Target nematode reference
COIF (f) COIR (r)	GATTTTTTGGKCATCCWGARG CWACATAATAAGTATCATG	He *et al.* (2005)	Partial *cox1*	*Xiphinema americanum sensu stricto*	He *et al.* (2005)
				X. americanum-group *Xiphinema*, *Longidorus*, *Paralongidorus*	Lazarova *et al.* (2006)
				Pratylenchus *Radopholus*	Palomares-Rius *et al.* (2017b)
				Hirschmanniella *Pratylenchoides*	Unpublished
COIF (f) XIPHR1 (r)	GATTTTTTGGKCATCCWGARG ACAATTCCAGTTAATCCTCCTACC	He *et al.* (2005), Lazarova *et al.* (2006)	Partial *cox1*	*Xiphinema, Longidorus, Paralongidorus*	Lazarova *et al.* (2006), Palomares-Rius *et al.* (2017b)
COIF (f) XIPHR2 (r)	GATTTTTTGGKCATCCWGARG GTACATAATGAAAATGTGCCAC	He *et al.* (2005), Lazarova *et al.* (2006)	Partial *cox1*	*Xiphinema, Longidorus, Paralongidorus*	Lazarova *et al.* (2006), Palomares-Rius *et al.* (2017b)
COIF5 (f) COIR9 (r)	AATWTWGGTGTTGGAACTTCTTGAAC CTTAAAACATAATGRAAATGWGCWAC WACATAATAAGTATC	Powers *et al.* (2014)	Partial *cox1*	Criconematidae *Hemicycliophora* Trichodoridae	Powers *et al.* (2014) van den Berg *et al.* (2018) Shaver *et al.* (2016)
JB3 (f) JB4.5 (r)	TTTTTTGGGCATCCTGAGGTTTAT TAAAGAAAGAACATAATGAAAATG	Bowles *et al.* (1992)	Partial *cox1*	*Pratylenchus* *Meloidogyne* *Scutellonema* *Rotylenchus* Longidoridae *Heterodera*	Janssen *et al.* (2017a) Janssen *et al.* (2017b) Kolombia *et al.* (2017) Cantalapiedra-Navarrete *et al.* (2013) Palomares-Rius *et al.* (2017b) De Luca *et al.* (2013)
JB3 (f) JB5 (r)	TTTTTTGGGCATCCTGAGGTTTAT AGCACCTAAACTTAAAACATAATGAAA	Bowles *et al.* (1992), Derycke *et al.* (2005)	Partial *cox1*	*Hoplolaimus* *Heterodera* *Meloidogyne*	Holguin *et al.* (2015) Toumi *et al.* (2013) Groover (2017)
COI-F5-Mel (f) COI-R9-Mel (r)	TGATTGATTTAGGTTCTGGAACTKSWTGAAC CATAATGAAAATGGGCAACAACATAATA AGTATC	Powers *et al.* (2018)	Partial *cox1*	*Meloidogyne*	Powers *et al.* (2018)
COI-F1 (f) COI-R2 (r)	CCTACTATGATTGGTGGTTTTGGTAATTG GTAGCAGCAGTAAAATAAGCACG	Kanzaki and Futai (2002)	Partial *cox1*	Parasitaphelenchidae Aphelenchoididae Anguinidae	Kanzaki and Futai (2002) Barrantes-Infante *et al.* (2018)

Continued

Table 14.1. Continued.

Primer combination[a]	Primer sequence (5′–3′)	Primer reference	DNA region	Target nematode	Target nematode reference
Het-coxiF (f)	TAGTTGATCGTAATTTTAATGG	Subbotin (2015)	Partial *cox1*	Heteroderidae	Subbotin (2015)
Het-coxiR (r)	CCTAAAACATAATGAAAATGWGC				
COX2-F (f)	GGACATCAGTGATATTGAAGATATG	Sultana *et al.* (2013b)	Partial *cox2*	*Bursaphelenchus* *Pratylenchus*	Sultana *et al.* (2013b)
COX2-R (r)	GCTACCTTAATGTCCTCACGCTAAG				
Cytb-424F (f)	GGWTAYGTWYTWCCWTGRGGWCARAT	von Nickisch-Rosenegk *et al.* (2001)	Partial *cob*	*Aphelenchoides*	Sun *et al.* (2014b)
Cytb-876R (r)	GCRTAWGCRAAWARRAARTAYCAYTCWGG				
CtybL14841 (f)	AAAAAGCTTCCATCCAACATC TCAGCATGATAAA	Kocher *et al.* (1989)	Partial *cob*	*Bursaphelenchus* *Pratylenchus*	Sultana *et al.* (2013b)
CtybH15149 (r)	AAACTGCAGCCCCTCAGAAT GATATTTGTCCTCA				
NAD1-F (f)	ACTYTDTAYGARCGTCATYTNYTRGG	Sun *et al.* (2014b)	Partial *nad1*	*Aphelenchoides*	Sun *et al.* (2014b)
NAD1-R (r)	CCWCTRACYARYTCHCTYTCHCCYTC				
NAD5F2 (f)	TATTTTTTGTTTGAGATATATTAG	Janssen *et al.* (2016)	Partial *nad5*	*Meloidogyne*	Janssen *et al.* (2016)
NAD5R1 (r)	CGTGAATCTTGATTTTCCATTTTT				
C2F3 (f)	GGTCAATGTTCAGAAATTTGTGG	Powers and Harris (1993)	*cox2-16S* rRNA	*Meloidogyne* *Scutellonema*	Powers and Harris (1993) Unpublished
1108 (r)	TACCTTTGACCAATCACGCT				
COI-F4a-Het (f)	CAGTTATATAATTCTTTTATTACTAGTCATG CATTAATTATRATTTTTTTTYTRGTTATACC	Powers *et al.* (2019)	Partial *cox1*	*Heterodera*	Powers *et al.* (2019)
COI-R10b-Het (r)	CCAAAAAAAAAAAAAATCACTATAATCYAAAT ATTTACGDGG				
COI-F7b-Prat (f)	GGDTGRACWTTHTAYCCNCC	Ozbayrak *et al.* (2019)	Partial *cox1*	*Pratylenchus*	Ozbayrak *et al.* (2019)
JB5 (r)	AGCACCTAAACTTAAAACATAATGAAAATG-3′	Derycke *et al.* (2005)			

[a] f, forward; r, reverse.

Prior to cloning and sequencing, PCR products can be isolated on an agarose gel (0.6% or 1%) using a QIAquick Gel Extraction Kit (Qiagen) and following the manufacturer's instructions.

14.4 Cloning and Sequencing

Purified PCR products can be used for primer-walking sequencing after confirming the sequences by direct sequencing reactions from both 5′ and 3′ ends. Since this requires a large amount of DNA, long PCR may not always provide high enough concentrations.

Purified PCR products (< 3 kb) generated with a proofreading DNA polymerase are cloned into a specific vector for products with blunt ends such as the Zero Blunt® TOPO® PCR Cloning Kit (Thermo Fisher Scientific) or treated with a *Taq* DNA polymerase to add an A-tail using the enzymatic method 'tailing'. Also, purified PCR products can be ligated into a pGEM-T Easy Vector using a pGEM-T Easy Vector Systems kit (Promega) and then transformed into *Escherichia coli* DH5α competent cells (or, alternatively, into One Shot TOP10 Electrocompetent *E. coli*) by electroporation (1.8 kV).

Transformed cells should then be cultured. Incubate the cells on LB (Luria-Bertani) agar plates with X-gal and IPTG at 37°C until colonies appear (usually more than 12 h). Move a single, full-grown white colony into a tube with LB, and incubate it in a shaking incubator for a minimum of 8 h. Extract plasmid DNAs using a QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's protocol.

Difficult or long DNA fragments (> 10 kb) should be cloned into a linear plasmid (e.g. pJAZZ) with the BigEasy PCR pJAZZ-OK Blunt Cloning Kit (Lucigen), following the manufacturer's instructions.

The sequences of the PCR-amplified and/or cloned DNA fragments can be determined with a Big Dye Terminator Cycle-Sequencing kit and an ABI PRISM 3730XL Analyzer (Applied Biosystem). If possible, long-PCR fragments can be sequenced from both directions using the primer-walking strategy. Some sequencing facilities offer a specific sequencing protocol for direct sequencing of difficult (secondary structures, GC rich fragments) or long templates.

Finally, assemble the entire mitochondrial DNA sequence by confirming the overlapping regions of the long-PCR fragments and the partial fragments.

14.5 Mitochondrial Genome Annotation and Gene Identification

The annotation of mt genomes can be performed in parts by using specialized websites or software to find protein-coding genes (PCGs), predict transfer and ribosomal RNAs and other structures. Also, a full annotation of the mt genome can be done automatically in a single web portal, such as DOGMA (Wyman *et al.*, 2004) and MITOS WebServers (Bernt *et al.*, 2013). (See Protocol 3 below for detailed information.)

Protein-coding genes can be searched using the Open Reading Frame Finder software at NCBI, with the invertebrate genetic code option. It is known that the stop codon of some PCGs overlap with the following neighbour gene (Plazzi *et al.*, 2013). Therefore, the first hypothetical truncated T/TA stop codon is annotated within the overlapping region. Transfer RNA (*trn*) genes and their cloverleaf secondary structures are identified using mainly two programs, tRNAscan-SE version 2.0 (Lowe and Chan, 2016) and MiTFi (mitochondrial transfer RNA finder) (Jühling *et al.*, 2012b). It is important to compare the outputs from the two programs and verify the anticodons manually. The *trnS* genes are identified manually by searching the anticodons. The MiTFi program can be implemented with the MITOS web server (Bernt *et al.*, 2013). The two ribosomal RNA genes *rrnL* and *rrnS* are identified by sequence comparisons with closely related nematodes.

Nematode mt genomes have tandem repetitive elements, inverted sequences and palindromes in the non-coding regions. Tandem repetitive elements can be identified using the Tandem Repeats Finder software (Benson, 1999) with the default parameters (*the basic option*). The inverted sequences and palindromes in the non-coding regions are detected with Einverted (http://www.bioinformatics.nl/cgi-bin/emboss/einverted) and Palindrome (http://www.bioinformatics.nl/cgi-bin/emboss/palindrome) programs available in the EMBOSS software package (Rice *et al.*, 2000) with default settings. Sequences from the different PCR fragments can be assembled using SnapGene® (GSL Biotech) (Figs 14.1 and 14.2) or Geneious (Biomatters Ltd).

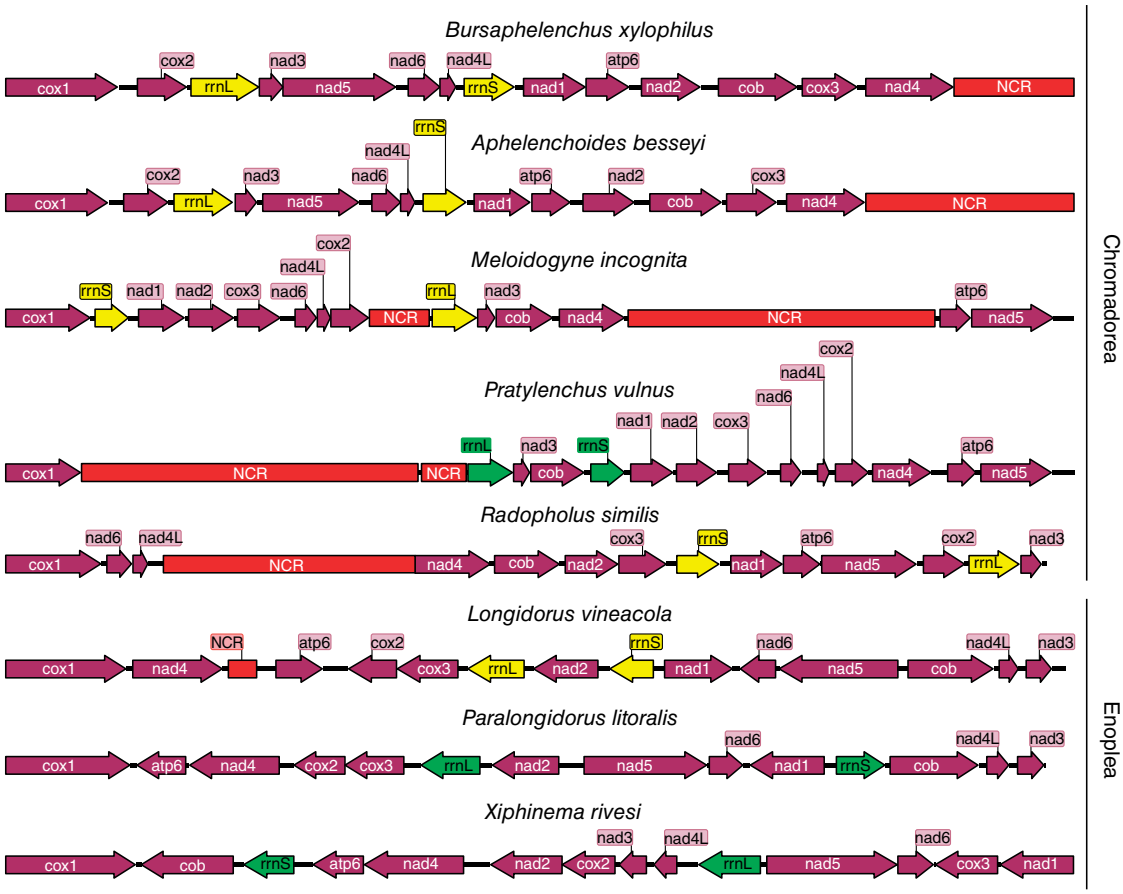

Fig. 14.2. Gene arrangement of the mitochondrial genomes of several plant-parasitic nematodes.

14.5.1 Protocols for mitochondrial genome annotation

Protocol 1: Prediction of transfer RNA genes secondary structures with tRNAscan-SE (Lowe and Chan, 2016)

- Access the tRNAscan-SE 2.0 web server http://lowelab.ucsc.edu/tRNAscan-SE/.
- Select **Other mitochondrial** in the sequence source option.
- Select **Default** in the search mode option.
- Choose either FASTA format or raw sequence.
- Paste your sequence on FASTA format/raw sequence or upload the FASTA file.
- Select **invertebrate mito** in the genetic code for tRNA isotype prediction options.
- Keep the default value on the score cutoff option.
- Click on Run **tRNAscan-SE**.

Protocol 2: Identification of tandem repeat elements with the Tandem Repeats Finder software (Benson, 1999)

- Access the software website https://tandem.bu.edu/trf/trf.html.
- Click on **Submit a Sequence for Analysis**.
- Select the option Basic, which will run the analysis under default parameters.
- Upload the sequence on FASTA format.
- Click on **Submit a Sequence**.

- Click on **Tandem Repeats Report**.
- Analyze the results.

Protocol 3: Mitochondrial genome annotation using the web-based annotation tool DOGMA (Wyman *et al.*, 2004)

- Format your sequence as a FASTA file.
- Go to **DOGMA** at http://dogma.ccbb.utexas.edu/ (Fig. 14.3).
- Create a user ID (click **Get Userid**).
- Fill out the information fields:
  - Userid: Your ID.
  - Unique identifier for the annotation (not your user ID).
  - Genome type: Choose the appropriate genome type for your sample.
  - Gapped alignment: Choose **Yes** or **No**.

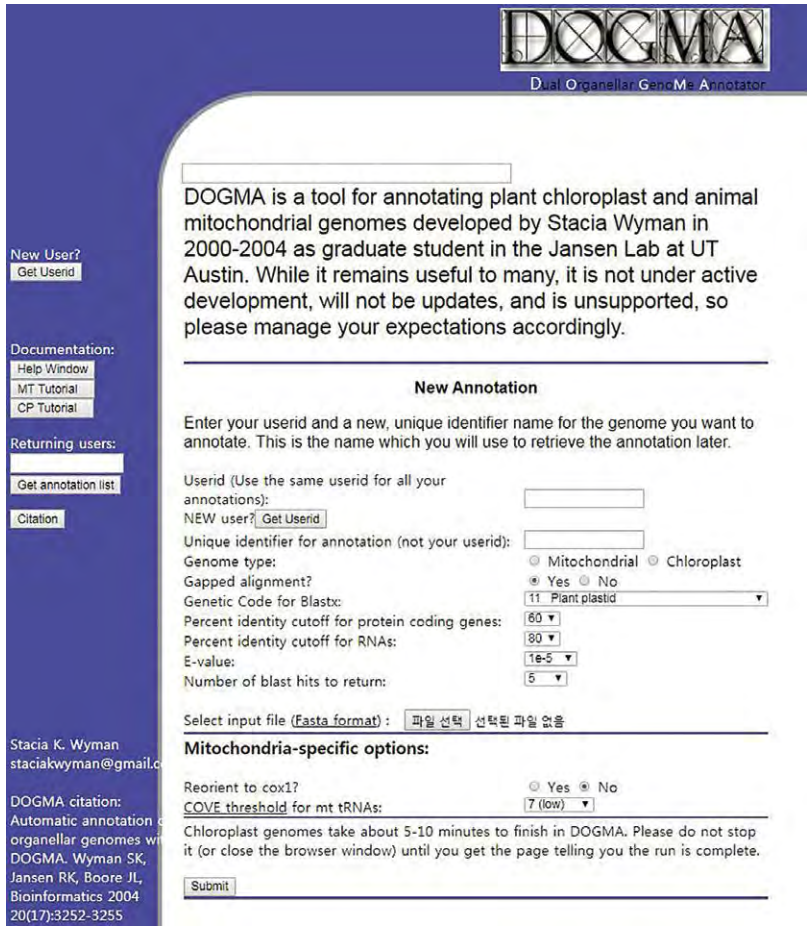

Fig. 14.3. Automatic annotation of mt genomes using the program DOGMA.

- Genetic Code for Blastx: Choose the appropriate genetic code for your sample.
- Etc: Choose appropriate options for your sample.

- Upload your FASTA file.
- Mitochondria-specific options: Choose options for your sample.
- Click **Submit**.

DOGMA results provide the inferred locations of genes in your sequence (Fig. 14.4). If you click on the bars that represent genes, you can see detailed information about each one.

- Protein-coding gene: Alignment results for amino acid sequences of homologous genes between your sample and related species (Fig. 14.5).

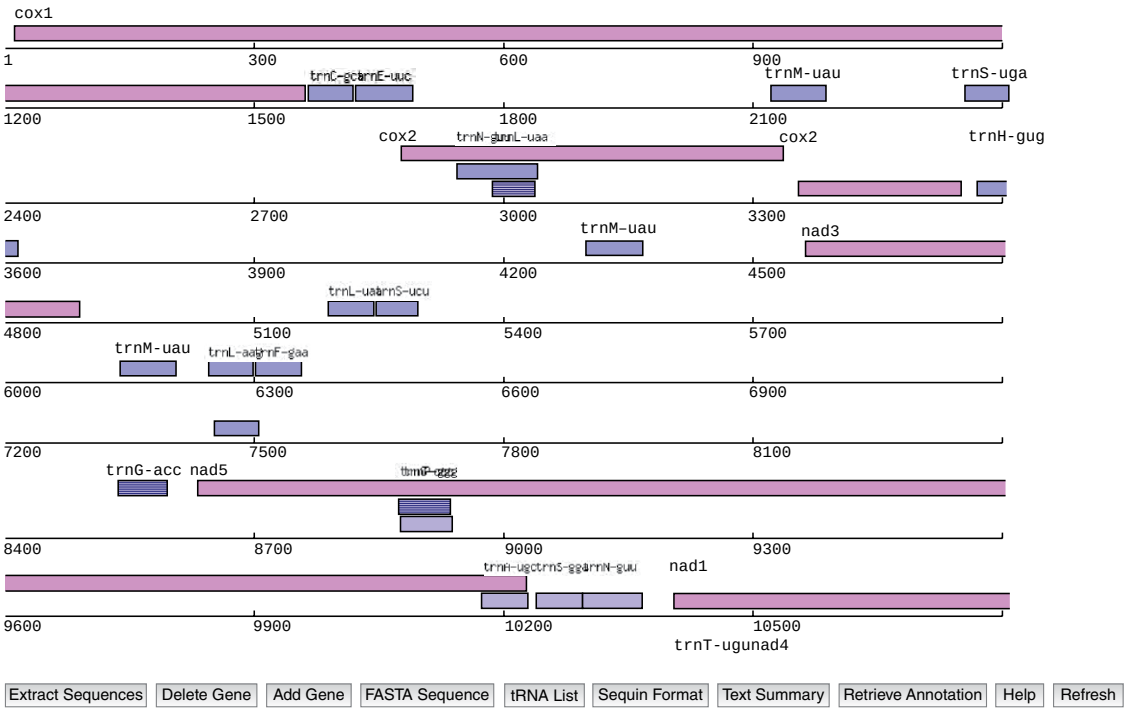

Fig. 14.4. DOGMA output showing the locations of the genes in the mt genome sequence.

Fig. 14.5. Amino acid sequences alignment of homologous genes.

- tRNA gene: Predicted secondary structure and DNA sequence information (Fig.14.6).

Protocol 4: Automated annotation of full mt genomes with MITOS (Bernt *et al.*, 2013)

- Format your sequence as a FASTA file.
- Go to the **MITOS WebServer** at http://mitos.bioinf.uni-leipzig.de/index.py (Fig.14.7).

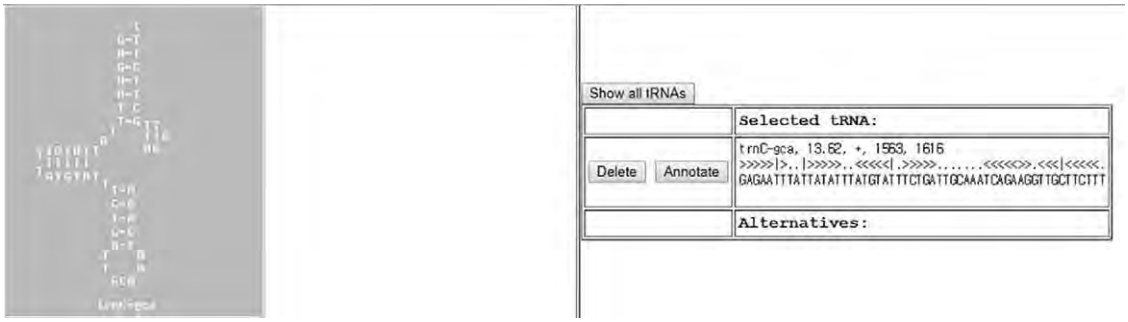

Fig. 14.6. Predicted secondary structure of the mt tRNA genes.

Fig. 14.7. Automatic annotation of mt genomes using the program MITOS.

- Fill out the information fields:
  - Name: Your name or ID.
  - E-mail: When the analysis is finished, you will be notified of the results and a link will be sent to your email address.
  - Job identifier: Create a unique name for this particular analysis.
  - Genetic Code: Choose the appropriate genetic code for your sample.
  - FASTA File: Upload your FASTA file.
- Click **Proceed**. Select the **Advanced** icon to choose additional options for the analysis.
- When the analysis is finished, you will receive an email.
- The MITOS result provides the inferred location, strand and length of each gene and the predicted secondary structure of tRNAs and rRNAs (Fig. 14.8).
- Predicted secondary structure images of tRNAs and rRNAs can be downloaded through the svg and ps icons in the **Structure** column.

Name	Start	Stop	Strand	Length	Structure
cox1	28	1461	+	1434	
trnC(tgc)	1573	1630	+	58	svg ps
rrnS	1599	2339	+	741	svg ps
trnS2(tca)	2347	2398	+	52	svg ps
cox2	2929	3549	+	621	
trnH(cac)	3561	3612	+	52	svg ps
rrnL	3987	4518	+	532	svg ps
nad3	4569	4880	+	312	
trnL2(tta)	5183	5237	+	55	svg ps
trnS1(aga)	5238	5287	+	50	svg ps
nad2	5288	6049	+	762	
trnN(aac)	6133	6187	+	55	svg ps
trnQ(caa)	6239	6291	+	53	svg ps
trnF(ttc)	6293	6349	+	57	svg ps
cob	6372	7412	+	1041	
trnL1(cta)	7453	7512	+	60	svg ps
cox3	7523	8260	+	738	
nad5	8679	9725	+	1047	
trnA(gca)	10171	10223	+	53	svg ps
trnP(cca)	10232	10288	+	57	svg ps
trnY(tac)	10339	10403	+	65	svg ps
nad1	10392	11255	+	864	
trnT(aca)	11602	11656	+	55	svg ps
nad4-0	11717	12793	+	1077	
trnM(atg)	12886	12944	+	59	svg ps
trnD(gac)	12945	13000	+	56	svg ps
nad4-2	12991	13044	-	54	
trnG(gga)	13274	13337	+	64	svg ps
atp6	13768	14178	+	411	
trnK(aaa)	14180	14241	+	62	svg ps
trnV(gta)	14241	14294	+	54	svg ps
nad6	14295	14723	+	429	
nad4l	14729	14962	+	234	
trnW(tga)	14963	15013	+	51	svg ps

tRNA gene rRNA gene protein coding gene

Fig. 14.8. MITOS output showing the location, strand and length of each mt gene.

14.5.2 Important information after automated annotation

Both MITOS or DOGMA websites predict the location of genes based on comparison with amino acid and nucleotide sequences of other organisms. However, all results must be manually checked as they may not accurately reflect unique characteristics of the taxon and gene sequence variation.

- Perform a BLAST analysis on GenBank and do sequence comparison in order to make sure that the sequence region proposed by MITOS or DOGMA is the same genetic region as the gene of related species.
- To confirm the initial and terminal codons of protein-coding genes, make comparisons with the amino acid sequences of genes of closely related species.
- Although the two rRNAs can be annotated by sequence comparison with other species, if the initial and/or terminal regions are unclear due to high sequence variation in the experimental species, the initial and terminal regions can be confirmed by inferring the secondary structure of the rRNAs.
- Both of the above programs indicate the expected tRNA region and predict its secondary structure. You should also check the anti-codon and secondary structure.
- Missed and unfit tRNAs can be found manually.
- The A+T-rich region is identified by the presence of general characteristics such as high A+T content, AT repeat block and DNA folding structure.

14.6 References

Armstrong, M.R., Blok, V.C. and Phillips, M.S. (2000) A multipartite mitochondrial genome in the potato cyst nematode *Globodera pallida*. *Genetics* 154, 181–192.

Balasubramanian, S. (2015) Solexa sequencing: Decoding genomes on a population scale. *Clinical Chemistry* 61, 21–24. DOI: 10.1373/clinchem.2014.221747

Barrantes-Infante, B.L., Schroeder, B.K., Subbotin, S.A. and Murray, T.D. (2018) *Afrina sporoboliae* sp. n. (Nematoda: Anguinidae) associated with *Sporobolus cryptandrus* from Idaho, United States: phylogenetic relationships and population structure. *Phytopathology* 108, 768–779. DOI: 10.1094/PHYTO-12-17-0395-R

Benson, G. (1999) Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Research* 27, 573–580. DOI: 10.1093/nar/27.2.573

Bernt, M., Donath, A., Jühling, F., Externbrink, F., Florentz, C., Fritzsch, G., Pütz, J., Middendorf, M. and Stadler, P.F. (2013) MITOS: Improved *de novo* metazoan mitochondrial genome annotation. *Molecular Phylogenetics and Evolution* 69, 313–319. DOI: 10.1016/j.ympev.2012.08.023

Besnard, G., Jühling, F., Chapuis, É., Zedane, L., Lhuillier, É., Mateille, T. and Bellafiore, S. (2014) Fast assembly of the mitochondrial genome of a plant parasitic nematode (*Meloidogyne graminicola*) using next generation sequencing. *Comptes Rendus Biologies* 337, 295–301. DOI: 10.1016/j.crvi.2014.03.003

Bowles, J., Blair, D. and McManus, D.P. (1992) Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Molecular and Biochemical Parasitology* 54, 165–173. DOI: 10.1016/0166-6851(92)90109-W

Cantalapiedra-Navarrete, C., Navas-Cortés, J.A., Liébanas, G., Vovlas, N., Subbotin, S.A., Palomares-Rius, J.E. and Castillo, P. (2013) Comparative molecular and morphological characterisations in the nematode genus *Rotylenchus: Rotylenchus paravitis* n. sp., an example of cryptic speciation. *Zoologischer Anzeiger – A Journal of Comparative Zoology* 252, 246–268. DOI: 10.1016/j.jcz.2012.08.002

De Luca, F., Vovlas, N., Lucarelli, G., Troccoli, A., Radicci, V., Fanelli, E., Cantalapiedra-Navarrete, C., Palomares-Rius, J.E. and Castillo, P. (2013) *Heterodera elachista* the Japanese cyst nematode parasitizing corn in Northern Italy: Integrative diagnosis and bionomics. *European Journal of Plant Pathology* 136, 857–872. DOI: 10.1007/s10658-013-0212-9

Derycke, S., Remerie, T., Vierstraete, A., Backeljau, T., Vanfleteren, J., Vincx, M. and Moens, T. (2005) Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. *Marine Ecology Progress Series* 300, 91–103. DOI: 10.3354/meps300091

García, L.E. and Sánchez-Puerta, M.V. (2015) Comparative and evolutionary analyses of *Meloidogyne* spp. based on mitochondrial genome sequences. *PLoS ONE* 10, e0121142. DOI: 10.1371/journal.pone.0121142

Gibson, T., Blok, V.C. and Dowton, M. (2007) Sequence and characterization of six mitochondrial subgenomes from *Globodera rostochiensis*: multipartite structure is conserved among close nematode relatives. *Journal of Molecular Evolution* 65, 308–315. DOI: 10.1007/s00239-007-9007-y

Gibson, T., Farrugia, D., Barrett, J., Chitwood, D.J., Rowe, J., Subbotin, S.A. and Dowton, M.P. (2011) The mitochondrial genome of the soybean cyst nematode, *Heterodera glycines*. *Genome* 54, 565–574. DOI: 10.1139/g11-024

Groover, W.L. (2017) *An evaluation of multiple techniques for the creation of a diagnostic tool for* Meloidogyne *species identification in Alabama*. M.Sc. Thesis, Auburn University, Auburn, Alabama, USA.

Gutiérrez-Gutiérrez, C., Castillo, P., Cantalapiedra-Navarrete, C., Landa, B.B., Derycke, S. and Palomares-Rius, J.E. (2011) Genetic structure of *Xiphinema pachtaicum* and *X. index* populations based on mitochondrial DNA variation. *Phytopathology* 101, 1168–1175. DOI: 10.1094/PHYTO-07-10-0194

He, Y., Jones, J., Armstrong, M., Lamberti, F. and Moens, M. (2005) The mitochondrial genome of *Xiphinema americanum sensu stricto* (Nematoda: Enoplea): considerable economization in the length and structural features of encoded genes. *Journal of Molecular Evolution* 61, 819–833. DOI: 10.1007/s00239-005-0102-7

Holguin, C.M., Baeza, J.A., Mueller, J.D. and Agudelo, P. (2015) High genetic diversity and geographic subdivision of three lance nematode species (*Hoplolaimus* spp.) in the United States. *Ecology and Evolution* 5, 2929–2944. DOI: 10.1002/ece3.1568

Humphreys-Pereira, D.A. and Elling, A.A. (2014) Mitochondrial genomes of *Meloidogyne chitwoodi* and *M. incognita* (Nematoda: Tylenchina): Comparative analysis, gene order and phylogenetic relationships with other nematodes. *Molecular and Biochemical Parasitology* 194, 20–32. DOI: 10.1016/j.molbiopara.2014.04.003

Humphreys-Pereira, D.A. and Elling, A.A. (2015) Mitochondrial genome plasticity among species of the nematode genus *Meloidogyne* (Nematoda: Tylenchina). *Gene* 560, 173–183. DOI: 10.1016/j.gene.2015.01.065

Humphreys-Pereira, D.A., Flores-Chaves, L., Gómez, M., Salazar, L., Gómez-Alpízar, L. and Elling, A.A. (2014) *Meloidogyne lopezi* n. sp. (Nematoda: Meloidogynidae), a new root-knot nematode associated with coffee *(Coffea arabica* L.) in Costa Rica, its diagnosis and phylogenetic relationship with other coffee-parasitizing *Meloidogyne* species. *Nematology* 16, 643–661. DOI: 10.1163/15685411-00002794

Jacob, J.E.M., Vanholme, B., Van Leeuwen, T. and Gheysen, G. (2009) A unique genetic code change in the mitochondrial genome of the parasitic nematode *Radopholus similis*. *BMC Research Notes* 2, 192. DOI: 10.1186/1756-0500-2-192

Janssen, T., Karssen, G., Verhaeven, M., Coyne, D. and Bert, W. (2016) Mitochondrial coding genome analysis of tropical root-knot nematodes *(Meloidogyne*) supports haplotype based diagnostics and reveals evidence of recent reticulate evolution. *Scientific Reports* 6, 22591. DOI: 10.1038/srep22591

Janssen, T., Karssen, G., Orlando, V., Subbotin, S.A. and Bert, W. (2017a) Molecular characterization and species delimiting of plant-parasitic nematodes of the genus *Pratylenchus* from the penetrans group (Nematoda: Pratylenchidae). *Molecular Phylogenetics and Evolution* 117, 30–48. DOI: 10.1016/j.ympev.2017.07.027

Janssen, T., Karssen, G., Topalović, O., Coyne, D. and Bert, W. (2017b) Integrative taxonomy of root-knot nematodes reveals multiple independent origins of mitotic parthenogenesis. *PLoS One* 12, e0172190. DOI: 10.1371/journal.pone.0172190

Jühling, F, Pütz, J., Florentz, C. and Stadler, P.F. (2012a) Armless mitochondrial tRNAs in Enoplea (Nematoda). *RNA Biology* 9, 1161–1166. DOI: 10.4161/rna.21630

Jühling, F, Pütz, J., Bernt, M., Donath, A., Middendorf, M., Florentz, C. and Stadler, P.F. (2012b) Improved systematic tRNA gene annotation allows new insights into the evolution of mitochondrial tRNA structures and into the mechanisms of mitochondrial genome rearrangements. *Nucleic Acids Research* 40, 2833–2845. DOI: 10.1093/nar/gkr1131

Jühling, T., Duchardt-Ferner, E., Bonin, S., Wöhnert, J., Pütz, J., Florentz, C., Betat, H., Sauter, C. and Mörl, M. (2018) Small but large enough: structural properties of armless mitochondrial tRNAs from the nematode *Romanomermis culicivorax. Nucleic Acids Research* 46, 9170–9180. DOI: 10.1093/nar/gky593

Kanzaki, N. and Futai, K. (2002) A PCR primer set for determination of phylogenetic relationships of *Bursaphelenchus* species within the *xylophilus* group. *Nematology* 4, 35–41. DOI: 10.1163/156854102760082186

Kiewnick. S., Holterman, M., van den Elsen, S., van Megen, H., Frey, J. and Helder, J. (2014) Comparison of two short DNA barcoding loci (COI and COII) and two longer ribosomal DNA genes (SSU and LSU rRNA) for specimen identification among quarantine root-knot nematodes (*Meloidogyne* spp.) and their close relatives. *European Journal of Plant Pathology* 140, 97–110. DOI: 10.1007/s10658-014-0446-1

Kim, J., Kern, E., Kim, T., Sim, M., Kim, J., Kim, Y., Park, C., Nadler, S.A. and Park, J.-K. (2017) Phylogenetic analysis of two *Plectus* mitochondrial genomes (Nematoda: Plectida) supports a sister group relationship between Plectida and Rhabditida within Chromadorea. *Molecular Phylogenetics and Evolution* 107, 90–102. DOI: 10.1016/j.ympev.2016.10.010

Kim, T., Kim, J., Cho, S., Min, G-S., Park, C., Carreno, R.A., Nadler, S.A. and Park, J.-K. (2014) Phylogeny of Rhigonematomorpha based on the complete mitochondrial genome of *Rhigonema thysanophora* (Nematoda: Chromadorea). *Zoologica Scripta* 43, 289–303. DOI:10.1111/zsc.12047

Kim, T., Kern, E., Park, C., Nadler, S.A., Bae, Y.J. and Park, J.-K. (2018) The bipartite mitochondrial genome of *Ruizia karukerae* (Rhigonematomorpha, Nematoda). *Scientific Reports* 8: 7482. DOI: 10.1038/s41598-018-25759-0

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V.S., Villa Blanca, F.X. and Wilson, A.C. (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences* 86, 6196–6200. DOI: 10.1073/pnas.86.16.6196

Kolombia, Y., Karssen, G., Viaene, N., Kumar, L., Joos, L., Coyne, D.L. and Bert, W. (2017) Morphological and molecular characterisation of *Scutellonema* species from yam (*Dioscorea* spp.) and a key to the species of the genus. *Nematology* 19, 751–787. DOI: 10.1163/15685411-00003084

Lavrov, D.V. and Brown, W.M. (2001) *Trichinella spiralis* mtDNA: a nematode mitochondrial genome that encodes a putative ATP8 and normally structured tRNAs and has a gene arrangement relatable to those of coelomate metazoans. *Genetics* 157, 621–637.

Lazarova, S.S., Malloch, G., Oliveira, C.M.G., Hübschen, J. and Neilson, R. (2006) Ribosomal and mitochondrial DNA analyses of *Xiphinema americanum*-group populations. *Journal of Nematology* 38, 404–410.

Liu, G.-H., Gasser, R.B., Su, A., Nejsum, P., Peng, L., Lin, R.-Q., Li, M.-W., Xu, M.-J. and Zhu, X.-Q. (2012a) Clear genetic distinctiveness between human- and pig-derived *Trichuris* based on analyses of mitochondrial datasets. *PLOS Neglected Tropical Diseases* 6, e1539. DOI: 10.1371/journal.pntd.0001539

Liu, G.-H., Wang, Y., Xu, M.-J., Zhou, D.-H., Ye, Y.-G., Li, J.-Y., Song, H.-Q., Lin, R.-Q. and Zhu, X.-Q. (2012b) Characterization of the complete mitochondrial genomes of two whipworms *Trichuris ovis* and *Trichuris discolor* (Nematoda: Trichuridae). *Infection, Genetics and Evolution* 12, 1635–1641. DOI: 10.1016/j.meegid.2012.08.005

Lowe, T.M. and Chan, P.P. (2016) tRNAscan-SE On-line: integrating search and context for analysis of transfer RNA genes. *Nucleic Acids Research* 44, W54–W57. DOI: 10.1093/nar/gkw413

Margulies, M., Egholm, M., Altman, W.E. *et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437, 376–380. DOI: 10.1038/nature03959

Okimoto, R. and Wolstenholme, D.R. (1990). A set of tRNAs that lack either the T psi C arm or the dihydrouridine arm: towards a minimal tRNA adaptor. *The EMBO Journal* 9, 3405–3411. DOI: 10.1002/j.1460-2075.1990.tb07542.x

Okimoto, R., Macfarlane, J.L., Clary, D.O. and Wolstenholme, D.R. (1992) The mitochondrial genomes of two nematodes, *Caenorhabditis elegans and Ascaris suum. Genetics* 130, 471–498.

Ozbayrak, M., Todd, T., Harris, T., Higgins, R., Powers, K., Mullin, P., Sutton, L. and Powers, T. (2019) A COI DNA barcoding survey of *Pratylenchus* species in the Great Plains Region of North America. *Journal of Nematology* 51, e2019-81. DOI: 10.21307/jofnem-2019-081

Palomares-Rius, J.E., Cantalapiedra-Navarrete, C., Archidona-Yuste, A., Subbotin, S.A. and Castillo, P. (2017a) The utility of mtDNA and rDNA for barcoding and phylogeny of plant-parasitic nematodes from Longidoridae (Nematoda, Enoplea). *Scientific Reports* 7, 10905. DOI: 10.1038/s41598-017-11085-4

Palomares-Rius, J.E., Cantalapiedra-Navarrete, C., Archidona-Yuste, A., Blok, V.C. and Castillo, P. (2017b) Mitochondrial genome diversity in dagger and needle nematodes (Nematoda: Longidoridae). *Scientific Reports* 7, 41813. DOI: 10.1038/srep41813

Phillips, W.S., Brown, A.M.V., Howe, D.K., Peetz, A.B., Blok, V.C., Denver, D.R. and Zasada, I.A. (2016) The mitochondrial genome of *Globodera ellingtonae* is composed of two circles with segregated gene content and differential copy numbers. *BMC Genomics* 17, 1–14. DOI: 10.1186/s12864-016-3047-x

Plantard, O., Picard, D., Valette, S., Scurrah, M., Grenier, E. and Mugniéry, D. (2008) Origin and genetic diversity of Western European populations of the potato cyst nematode (*Globodera pallida*) inferred from mitochondrial sequences and microsatellite loci. *Molecular Ecology* 17, 2208–2218. DOI: 10.1111/j.1365-294X.2008.03718.x|

Plazzi, F., Ribani, A. and Passamonti, M. (2013) The complete mitochondrial genome of *Solemya velum (*Mollusca: Bivalvia) and its relationships with conchifera. *BMC Genomics* 14, 409. DOI: 10.1186/1471-2164-14-409

Powers, T.O. and Harris, T.S. (1993) A polymerase chain reaction method for identification of five major *Meloidogyne* species. *Journal of Nematology* 25, 1–6.

Powers, T.O., Mullin, P.G., Harris, T.S., Sutton, L.A. and Higgins, R.S. (2005) Incorporating molecular identification of *Meloidogyne* spp. into a large-scale regional nematode survey. *Journal of Nematology* 37, 226–235.

Powers, T.O., Bernard, E.C., Harris, T., Higgins, R., Olson, M., Lodema, M., Mullin, P., Sutton, L. and Powers, K.S. (2014) COI haplotype groups in *Mesocriconema* (Nematoda: Criconematidae) and their morphospecies associations. *Zootaxa* 3827, 101–146. DOI: 10.11646/zootaxa.3827.2.1

Powers, T.O., Harris, T., Higgins, R., Mullin, P. and Powers, K. (2018) Discovery and identification of *Meloidogyne* species using COI DNA barcoding. *Journal of Nematology* 50, 399–412.

Powers, T., Skantar, A., Harris, T., Higgins, R., Mullin, P., Hafez, S., Handoo, Z., Todd, T. and Powers, K. (2019) DNA barcoding evidence for the North American presence of alfalfa cyst nematode, *Heterodera medicaginis*. *Journal of Nematology* 51, e2019-16. DOI 10.21307/jofnem-2019-016

Rice, P., Longden, I. and Bleasby, A. (2000) EMBOSS: the European molecular biology open software suite. *Trends in Genetics* 16, 276–277. DOI: 10.1016/S0168-9525(00)02024-2

Shaver, B.R., Marchant, S., Martin, S.B. and Agudelo, P. (2016) 18S rRNA and COI haplotype diversity of *Trichodorus obtusus* from turfgrass in South Carolina. *Nematology* 18, 53–65. DOI: 10.1163/15685411-000029

Shendure, J. and Ji, H. (2008) Next-generation DNA sequencing. *Nature Biotechnology* 26, 1135–1145. DOI: 10.1038/nbt1486

Subbotin, S.A. (2015) *Heterodera sturhani* sp. n. from China, a new species of the *Heterodera avenae* species complex (Tylenchida: Heteroderidae). *Russian Journal of Nematology* 23, 145–152.

Subbotin, S.A., Toumi, F., Elekçioğlu, I.H., Waeyenberge, L. and Tanha Maafi, Z. (2018). DNA barcoding, phylogeny and phylogeography of the cyst nematodes of the *Avenae* group from the genus *Heterodera* (Tylenchida: Heteroderidae). *Nematology* 20, 671–702. DOI: 10.1163/15685411-00003170

Sultana, T., Kim, J., Lee, S.-H., Han, H., Kim, S., Min, G.-S., Nadler, S.A. and Park, J.-K. (2013a) Comparative analysis of complete mitochondrial genome sequences confirms independent origins of plant-parasitic nematodes. *BMC Evolutionary Biology* 13, 12. DOI: 10.1186/1471-2148-13-12

Sultana, T., Han, H. and Park, J.-K. (2013b) Comparison of complete mitochondrial genomes of pine wilt nematode *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus* (Nematoda: Aphelenchoidea) and development of a molecular tool for species identification. *Gene* 520, 39–46. DOI: 10.1016/j.gene.2013.02.006

Sun, L., Zhuo, K., Lin, B., Wang, H. and Liao, J. (2014a) The complete mitochondrial genome of *Meloidogyne graminicola* (Tylenchina): a unique gene arrangement and its phylogenetic implications. *PLoS One* 9, e98558. DOI: 10.1371/journal.pone.0098558

Sun, L., Chi, W., Zhuo, K., Song, H., Zhang, L.-H., Liao, J. and Wang, H. (2014b) The complete mitochondrial genome of *Aphelenchoides besseyi* (Nematoda: Aphelenchoididae), the first sequenced representative of the subfamily Aphelenchoidinae. *Nematology*, 16, 1167–1180. DOI: 10.1163/15685411-00002844

Toumi, F., Waeyenberge, L., Viaene, N., Dababat, A., Nicol, J.M., Ogbonnaya, F. and Moens, M. (2013) Development of two species-specific primer sets to detect the cereal cyst nematodes *Heterodera avenae* and *Heterodera filipjevi. European Journal of Plant Pathology* 136, 613–624. DOI: 10.1007/s10658-013-0192-9

Van den Berg, E., Tiedt, L.R., Liebanas, G., Chitambar, J.J., Stanley, J.D., Inserra, R.N., Castillo, P. and Subbotin, S.A. (2018) Morphological and molecular characterisation of two new *Hemicycliophora* species (Tylenchida: Hemicycliophoridae) with a revision of the taxonomic status of some known species and a phylogeny of the genus. *Nematology* 20, 319–354. DOI: 10.1163/15685411-00003143

von Nickisch-Rosenegk, M., Brown, W.M. and Boore, J.L. (2001) Complete sequence of the mitochondrial genome of the tapeworm *Hymenolepis diminuta*: Gene arrangements indicate that platyhelminths are eutrochozoans. *Molecular Biology and Evolution* 18, 721–730. DOI: 10.1093/oxfordjournals.molbev.a003854

Watanabe, Y., Tsurui, H., Ueda, T., Furushima, R., Takamiya, S, Kita, K., Nishikawa, K. and Watanabe, K. (1994) Primary and higher order structures of nematode (*Ascaris suum*) mitochondrial tRNAs lacking either the T or D stem. *The Journal of Biological Chemistry* 269, 22902–22906

Wolstenholme, D.R., Macfarlane, J.L., Okimoto, R., Clary, D.O. and Wahleithner, J.A. (1987) Bizarre tRNAs inferred from DNA sequences of mitochondrial genomes of nematode worms. *Proceedings of the National Academy of Sciences of the United States of America* 84, 1324–1328. DOI: 10.1073/pnas.84.5.1324

Wyman, S.K., Jansen, R.K. and Boore, J.L. (2004) Automatic annotation of organellar genomes with DOGMA. *Bioinformatics* 20, 3252–3255. DOI: 10.1093/bioinformatics/bth352

15 Phylogenetic Analysis of DNA Sequence Data

SERGEI A. SUBBOTIN

Plant Pest Diagnostic Center, California Department of Food and Agriculture, California, USA; Center of Parasitology of A.N. Severtsov Institute of Ecology and Evolution, Moscow, Russia

15.1 Introduction

The goal of phylogenetics is to construct relationships that are true representations of the evolutionary history of a group of organisms or genes. The history inferred from phylogenetic analysis is usually depicted as branching in tree-like diagrams or networks. In nematology, phylogenetic studies have been applied to resolve a wide range of questions dealing with improving classifications and testing evolution processes, such as co-evolution, biogeography and many others. There are several main steps involved in a phylogenetic study:

- selection of ingroup and outgroup taxa for a study;
- selection of one or several gene fragments for a study;
- sample collection, obtaining PCR products and sequencing of gene fragments;
- visualization, editing raw sequence data and sequence assembling;
- search for sequence similarity in a public database;
- making and editing multiple alignment of sequences;
- selecting appropriate DNA model for a dataset;
- phylogenetic reconstruction using minimum evolution, maximum parsimony, maximum likelihood and Bayesian inference;
- visualization of tree files and preparation of tree for a publication; and
- sequence submission to a public database.

Molecular phylogenetic study requires particularly careful planning because it is usually relatively expensive in terms of the cost in reagents and time. The first and the most important step of any study is to define clearly the specific biological question to be answered. When the biological problem is formulated and the

 Techniques for Work with Plant and Soil Nematodes (eds R.N. Perry, D.J. Hunt and S.A. Subbotin)
10.1079/9781786391759.0015

literature survey pertaining to the group of interest is completed, selection of ingroup and outgroup taxa and appropriate genes should be done. These could be representatives of the same species from various locations or hosts, or different species of the same genus, or representatives of related genera or higher taxonomic categories. It is advisable to include as many samples as possible, as well as to choose several gene fragments to reduce artefactual associations between terminals.

The third stage, which includes sample collection, obtaining PCR products and sequencing, may require several weeks or months and is the most time-consuming stage in a study. Before launching a full-scale project, a pilot study with a limited sample number is recommended to determine if the selected genetic markers give sufficient resolution for phylogeny of the studied group.

The last seven stages require a computer with an Internet connection and suitable software. This chapter deals with these stages. The software and instructions for its use are discussed below. All of them are free and can be downloaded from different websites, except for PAUP* (Phylogenetic Analysis Using Parsimony*), which can be purchased from the official website. This chapter only considers data from nucleotide sequences and does not take into account amino acid sequences data.

There are many phylogenetics programs that perform similar functions (Table 15.1). The comprehensive list can be found in the websites:

- http://evolution.genetics.washington.edu/phylip/software.html;
- http://www.phylo.org;
- https://www.phylogeny.fr;
- https://isogg.org/wiki/Phylogeny_programs;
- https://en.wikipedia.org/wiki/List_of_phylogenetics_software; and
- https://molbiol-tools.ca/Phylogeny.htm.

Table 15.1. Phylogenetics programs.

Software	Website
BEAST (**B**ayesian **E**volutionary **A**nalysis **S**ampling **T**rees) is a cross-platform program for Bayesian phylogenetic analysis of molecular sequences using MCMC	https://beast.community https://www.beast2.org
BioEdit is a **Bio**logical Sequence **Edit**or	https://bioedit.software.informer.com
Clustal is a series of widely used computer programs used in Bioinformatics for multiple sequence alignment	http://www.clustal.org
GARLI (**G**enetic **A**lgorithm for **R**apid **L**ikelihood **I**nference) is a program for inferring phylogenetic trees	https://code.google.com/archive/p/garli/
MAFFT (**M**ultiple **A**lignment using **F**ast **F**ourier **T**ransform) is a program used to create multiple sequence alignments of amino acid or nucleotide sequences.	https://mafft.cbrc.jp/alignment/software/
MEGA (**M**olecular **E**volutionary **G**enetic **A**nalysis) is a software for conducting statistical analysis of molecular evolution and for constructing **phylogenetic** trees.	https://www.megasoftware.net
MrBayes is a free software tool that performs Bayesian inference of phylogeny	https://nbisweden.github.io/MrBayes/
MUSCLE (**Mu**ltiple **S**equence **C**omparison by **L**og-**E**xpectation) is a software for multiple sequence alignment of protein and nucleotide sequences.	http://www.drive5.com/muscle/
PAUP* - **P**hylogenetic **A**nalysis **U**sing **P**arsimony (*and other methods)	http://paup.phylosolutions.com
RAxML (**R**andomized **Ax**elerated **M**aximum **L**ikelihood) is a program for sequential and parallel maximum likelihood-based inference of large phylogenetic trees.	https://cme.h-its.org/exelixis/web/software/raxml/index.html http://www.trex.uqam.ca/index.php?action=raxml&project=trex
T-Coffee (**T**ree-based **C**onsistency **O**bjective **F**unction **f**or Alignm**e**nt **E**valuation) is a multiple sequence alignment software using a progressive approach	http://www.tcoffee.org

The reliability and practicality of the software depends on the structure and size of the data. The merits and pitfalls of various methods are the subject of often acrimonious debates in taxonomic and phylogenetic journals (Lemey *et al.*, 2009; Yang and Rannala, 2012). Generally, most software packages have been developed and maintained through the efforts of scientists in related fields and released under free software licences.

Phylogenetics is a rather complex and rapidly expanding field of research. In this chapter, some basic approaches for the analysis of nucleotide non-coding gene sequences are given and discussed with an assumption that the gene tree reflects the organism phylogeny. Many other important software with statistical testing for phylogenetic studies are not covered here, and specialized literature (e.g. Lemey *et al.*, 2009; Hall, 2017) may be recommended for researchers.

15.2 Visualization and Editing of Raw Sequence Data

The sequencer is a laser-based instrument that utilizes fluorescent labels to analyse the products of a sequencing reaction as they migrate through a gel. After the data are collected from a sequencing run, special software identifies and tracks the sample lanes of the gel and subsequently normalizes and integrates the data into files. Automated DNA sequencer generates two file types: (i) a four-colour chromatogram showing the results of the sequencing run; and (ii) a text file of sequence data. It is always highly recommended to check the quality of the chromatogram file of a studied sample before converting it to a sequence text file.

There are several programs for visualization of a raw sequence data, two of which can be freely downloaded: Chromas (http://technelysium.com.au/wp/chromas/) developed by Technelysium Pty Ltd and FinchTV (http://www.geospiza.com/ftvdlinfo.html) originally designed by Geospiza. Chromas is a simple, easy-to-use viewer and editor for chromatograms from automated Sanger sequencers. Chromas has several features including automatic removal of low-quality sequence or vector sequences, copying the sequence to the clipboard in plain text, FASTA format for pasting into other applications, performing reverse and complement the sequence and chromatogram, and displaying translations in three frames along with the sequence. A chromatogram shows a sequence of peaks in four colours, each representing the base: A, green or yellow; G, black; T, red; and C, blue.

Once the sequence is obtained, the quality of sequence reading should be proofread to ensure that all ambiguous sites are correctly resolved in a chromatogram file. Good-quality sequences are characterized by well-defined peak resolution, uniform peak spacing and high signal-to-noise ratios (Fig. 15.1). If the quality of a sequence is not good and contains double or asymmetrical peaks or strong background noise, sequencing reactions should be repeated with both forward and reverse primers (Fig. 15.2). There are many reasons why a sequence reaction can fail. A good sequence generally begins approximately around 20–30 bp and lasts up to 700–800 bp. Any bad-quality sequence should be eliminated from further analysis. Sometimes, the software may miscall or miss a nucleotide or add an additional nucleotide, making proofreading of chromatograph files an important step of data verification. A single peak position within a trace may have also two peaks of different colours instead of just one. This is a common problem when sequencing a PCR product derived

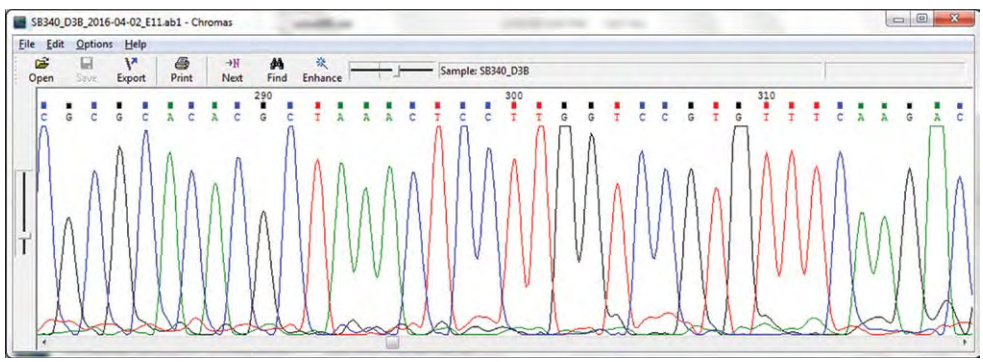

Fig. 15.1. Chromas with good-quality sequence containing well-defined peaks.

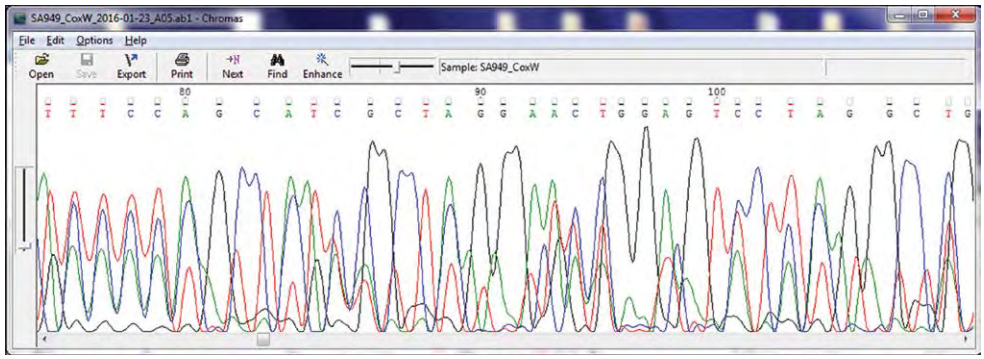

Fig. 15.2. Chromas with bad-quality sequence containing double peaks and strong background.

Fig. 15.3. Sequence in FASTA format.

from diploid genomic DNA having polymorphic positions. In this case, it is recommended to edit the base in a chromatogram file according to the single letter nucleic acid code recommendations: R, G or A (purine); Y, T or C (pyrimidine); K, G or T (keto); M, A or C (amino); S, G or C (strong); W, A or T (weak); B, G or T or C; D, G or A or T; H, A or C or T; V, G or C or A; N, A or G or C or T (any).

Work with Chromas version 2.62 includes a few steps:

- Open a chromatogram file. Choose **File > Open**.
- If an antisense chain (3′–5′) is open, use reverse and complement option. Choose **Edit > Reverse+Complement**.
- Check quality and trim low-quality data. Choose **Edit > Trim Low Quality.**
- Convert into a FASTA format, which begins with a single-line description, followed by lines of sequence data. Choose **File > Export**. After a chromatogram file has been examined and edited, it should be exported into a FASTA format file (Fig. 15.3).

15.3 Sequence Assembling

If a PCR product cannot be covered by a single sequencing reaction, several reactions should be performed. The chromatograms of these reactions should be verified and then displayed in a single FASTA format file. The goal of assembling is to create a single consensus sequence covering the whole length of a studied amplicon from several partly overlapped sequences.

A number of DNA sequence assembly programs have been developed including CAP3, which has also web-based version (http://doua.prabi.fr/software/cap3). For more advanced usage, it is recommended to install the software on your local computer. The program features include fast identification of pairs of reads

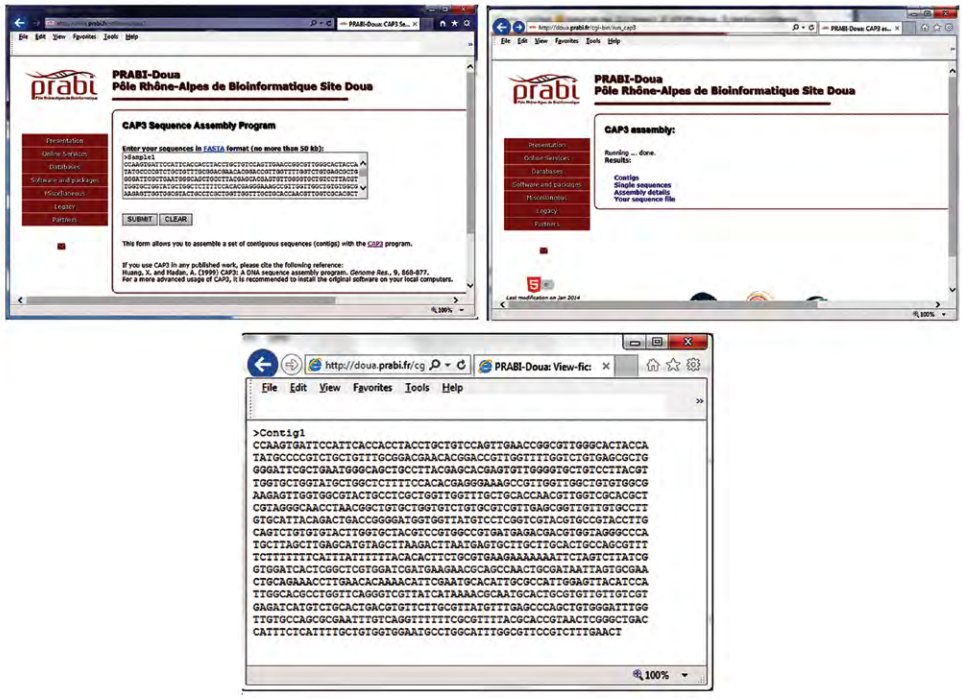

Fig. 15.4. CAP3 home page and output.

with an overlap, clipping of 5′ and 3′ poor regions of reads, efficient computation and evaluation of overlaps, use of forward–reverse constraints to correct errors in construction of a contig, and generation of consensus sequence for a contig (Huang and Madan, 1999).

Work with web-based CAP3 is simple and the results are self-explanatory.

- Enter your sequences in FASTA format in a window and submit the data (Fig. 15.4).
- Results are displayed as contigs, single sequence, assembling details and your sequence file. Every base in an assembly must be covered by at least two sequences of high quality. Validating sequence coverage provides a high degree of confidence in the consensus base calls.

15.4 Similarity Search in a Sequence Database

One of the most important steps in the study is the identification of your sequence and comparison with all known sequences collected in different databases. This procedure is called similarity search. BLAST (Basic Local Alignment Search Tool) is a powerful program for rapid searching of nucleotide and protein databases. The BLAST program finds regions of local similarity between sequences. It was developed in 1989 at the National Center for Biotechnology Information (NCBI), Maryland, USA. A BLAST query uses statistical methods to compare a DNA or protein input sequence ('query sequence') to a database of sequences ('subject sequences') and returns those sequences that have a significant level of similarity to the query sequence. The BLAST algorithm calculates similarity scores for local alignments (i.e. the most similar regions between two sequences) between the query sequence and subject sequences using specific scoring matrices, and returns a table of the best matches ('hits') from the database (Altschul, 1990; Wheeler and Bhagwat, 2007).

A BLAST search includes a few steps:

- Point your browser to the NCBI BLAST server at: http://www.ncbi.nlm.nih.gov/BLAST.
- Select **Nucleotide BLAST** (Fig. 15.5).

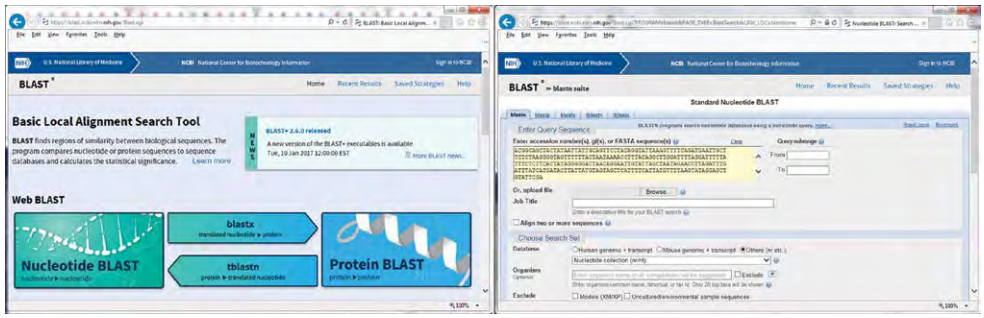

Fig. 15.5. BLAST searching pages.

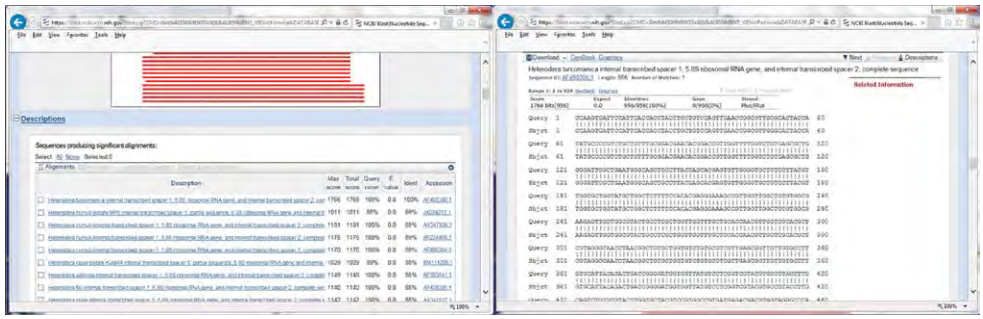

Fig. 15.6. BLAST output pages.

- Paste your sequence in the Query Sequence field, choose search set in the Database as **Others, Nucleotide collection. Organism group** could be also defined to limit your search to the DNA of a specific organism. Alignment parameters can be also modified (for example, increase: **Max target sequences**). The default setting uses a version of BLASTN called **megaBLAST**. Click the **BLAST** button and wait for the results.

The results from a BLAST search are divided into three sections: (i) the graphic pane; (ii) a results table; and (iii) the alignments between the query and the hits. Conclusions can be based on interpretations of the BLAST results table that provides basic information about the hits together with the statistics of each hit. Information includes: Max score (highest alignment score (bit-score) between the query sequence and the database sequence segment); Total score (sum of alignment scores of all segments from the same database sequence that match the query sequence (calculated over all segments)); Query cover (the percentage of the query that aligns with the hit); E value (number of alignments expected by chance with a particular score or better); Identity (percentage of identity between the query and the hit in a nucleotide to nucleotide alignment); and Accession (GenBank sequence number). The top hits are most significant and similar to the submitted sequence (Fig. 15.6).

15.5 Sequence Retrieval from the Database

There are several ways to retrieve sequences to build your dataset for phylogenetic analysis:

- Sequences can be obtained from the BLAST search result page. Click on **Accession** and the hyperlink takes you to the database entry that contains this sequence. Click on **FASTA** to convert the GenBank format into a FASTA format (Fig. 15.7).
- Point your browser to http://www.ncbi.nlm.nih.gov. Select **Nucleotide** in a search field and type organism name(s) and gene name or accession number(s).

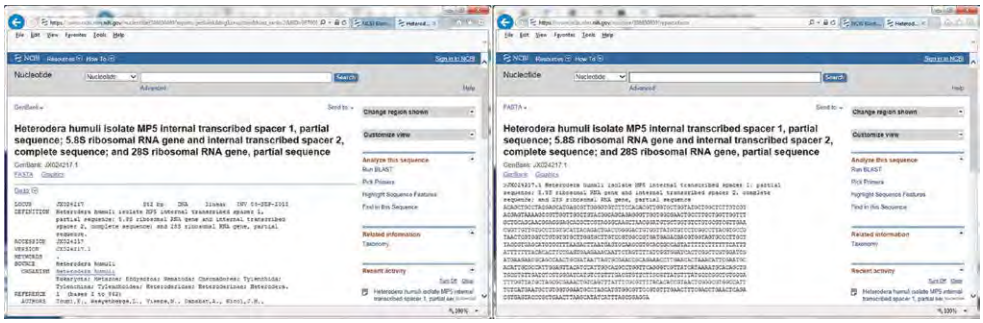

Fig. 15.7. GenBank entry JX024217.

15.5.1 Multiple alignment

The construction of alignment is the establishment of positional homology between nucleotides or amino acid bases that have descended from a common ancestral base. Errors incurred in this step can lead to an incorrect phylogeny. Multiple alignment construction is an exclusively mathematical process and is usually constructed using computer programs with particular algorithms. Most alignments are made based only on sequence information. However, aligning according to secondary structure is considered more reliable than sequence-based alignment because confidence in homology assessment is greater when comparisons are made based on structures rather than on simple characters. This approach is time consuming, requires information on secondary structure and can be done only with special alignment software.

Clustal (http://www.clustal.org) is the most popular multiple sequence alignment program and one of the most widely cited scientific publication (Larkin *et al.*, 2007). There are two main variations: ClustalW with command line interface ('W' stands for 'weighting' different parts of alignment differently) and ClustalX with a graphical user interface. Alignment can be made using web-based Clustal or software downloaded onto a computer. Clustal performs a global-multiple sequence alignment by the progressive method using a three-step process: (i) perform pairwise alignment of all the sequences by dynamic programming; it aligns each sequence against each other giving a similarity matrix; (ii) create a guide dendrogram using the similarity matrix; and (iii) start by aligning the two most similar sequences. Following the guide dendrogram, the next sequences are added in, aligning them to the existing alignment and insert gaps as necessary.

- Double click on ClustalX icon and run the program. Load sequence the sequence file. Choose **File > Load Sequences.** Under the alignment menu, choose the output format options.
- Under this menu, it is possible to change the alignment parameters (Gap Opening Penalty, Gap Extension Penalty), both for pairwise alignment and for the multiple alignment stages or run with default options.
- Choose **Alignment > Do Complete Alignment** and click **OK.** The sequence alignment is displayed in a window on the screen. The histogram below the ruler indicates the degree of similarity (Fig. 15.8).
- Clustal generates two output files, with extension 'aln' (alignment result in Clustal format) and extension 'dnd' (guide dendrogram).

It is recommended to perform phylogenetic analyses based on a series of slightly modified alignments to determine how ambiguous regions in the alignment affect the results.

In addition to Clustal, other software can be used to make alignments (Table 15.1).

15.5.2 Sequence alignment editing

Any automatic alignment should be visually checked and then manually edited, if necessary. GeneDoc (https://genedoc.software.informer.com) is a multiple sequence editor with a full-featured alignment visualization

including shading and structural definition features, editing and analysis tools. It has an easy-to-use point and click user interface with extensive keyboard mapping for advanced users (Nicholas *et al.*, 1997).

- **Run Genedoc**. To import the file generated by Clustal, choose **File > New > Import**. In Import Type window, select **Clustal** (**ALN**) and click **>Import**. In Open window, find your file and click**>** **Open** and then **Done**. The alignment will be displayed (Fig. 15.9).
- To see the full sequence names in the displayed alignment: use combination **Ctrl+G** or choose **Project > Configure** and then in the field 'Max NemLen' type '30' **> OK.**
- There are many features to edit alignment. To edit sequence list, use combination **Ctrl+Q** or select **Project > Edit Sequence list**. To move nucleotide(s) or insert gap(s), use **Grab+Drag bottom** (**Ctrl+A**), **Grab+Slide bottom** (**F5**) Insert/Delete gap in single sequence (**F6**), **Insert Gap Colum** (**F7**)**.** To replace nucleotide, use **Ctrl+U** or **Edit > Residue Edit Mode.**
- To display the alignment with only nucleotide differences, use the combination **Ctrl+G** or choose **Project > Configure,** select **Shade** and in **Residue Display Mode > Differences** and in **Difference Mode Style > Diff/ Top Sequence,** click **OK.**

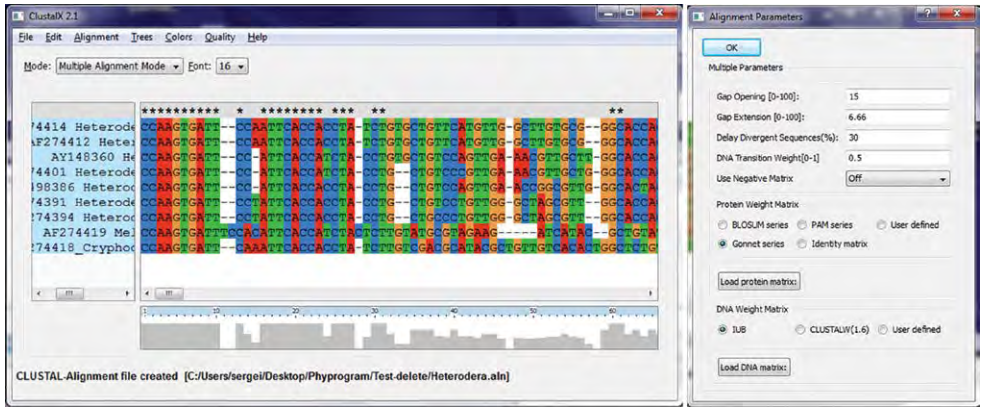

Fig. 15.8. A multiple sequence alignment generated with ClustalX and alignment parameters.

Fig. 15.9. A multiple sequence alignment in GenDoc.

- To trim excess sequence in the beginning and end of alignment or remove any alignment region: use combination **Ctrl+L** or click **Edit,** select **> Select Columns** and then use the cursor to select alignment region. To delete this region: use combination **Ctrl+D,** or click **Edit,** select **> Delete All Data.**
- To prepare the alignment file for a publication, use combination **Ctrl+E** or **Edit > Select Blocks for Copy,** and use a cursor to mark block(s). When all blocks are marked, click **Edit > Copy Selected Blocks** to **> RTF file**, then **Save as**. The saved file could be opened in Word.
- To save the file use **Ctrl+S** or **File > Save.** Export the corrected GenDoc file into a FASTA format, choose **File > Export,** then type the file name, click on **Save > Done**. The result will be saved.

Multiple alignment can also be automatically edited using the computer program Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks.html) that eliminates poorly aligned positions and divergent regions of an alignment of DNA or protein sequences (Castresana, 2000; Talavera and Castresana, 2007). These positions may not be homologous or may have been saturated by multiple substitutions and it is convenient to eliminate them prior to phylogenetic analysis. Gblocks selects blocks in a similar way as done by hand but following a reproducible set of conditions. The selected blocks must fulfil certain requirements with respect to the lack of large segments of contiguous non-conserved positions, lack of gap positions and high conservation of flanking positions, making the final alignment more suitable for phylogenetic analysis. Gblocks is very fast in processing alignments and it is therefore highly suitable for large-scale phylogenetic analyses. Gblocks can be run on a computer and run on web: http://molevol.cmima.csic.es/castresana/Gblocks_server.html.

- Go to Gblocks web-server. Insert the alignment in a FASTA format in a window and select options.
- Click on **Get Block** bottom. Gblocks outputs files to visualize the selected blocks (Fig. 15.10).
- Click on **Resulting alignment** to see the result.

15.6 File Format Converting

Each phylogenetic program requires an alignment prepared in certain file format. Several popular phylogenetic programs such as PAUP*, MrBayes, Mesquite, MacClade and others use the NEXUS format widely used in bioinformatics. ForCon is a user-friendly software tool (http://bioinformatics.psb.ugent.be/webtools/ForCon/)

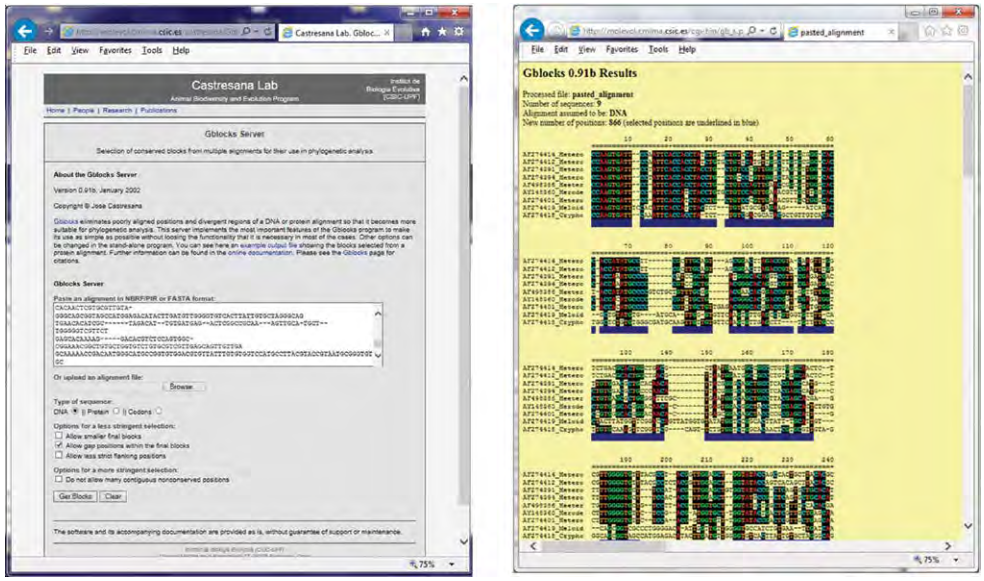

Fig. 15.10. Gblock Server home page and output.

Fig. 15.11. ForCon windows.

for the conversion of nucleic acid and amino acid sequence alignments. ForCon is able to convert in both ways, i.e. reading and writing.

- Run ForCon program. Choose **Enter,** then select Input format > **FASTA** and for Output format >**PAUP/NEXUS(i)** > OK (Fig. 15.11).
- In Open window, find your file in FASTA format > **Open** > **OK.** In **Save as** window, **Type** file name with file extension 'nex' > **Save.** Then choose **Select All > OK.**

The file conversion could also be done via different on-line tools, for example, http://www.ebi.ac.uk/Tools/sfc/emboss_seqret/ and https://hcv.lanl.gov/content/sequence/FORMAT_CONVERSION/form.html.

15.7 Selection of Model of Sequence Evolution

When using minimum evolution (distance), maximum likelihood methods or Bayesian inference to build trees, it needs to find a model of sequence evolution that fits the DNA changes in the aligned sequences that are being used. The substitution models differ in terms of the parameters used to describe the rates at which one nucleotide replaces another during evolution. The best-fit substitution model can be selected using the Modeltest program (Posada and Crandall, 1998), MrModeltest (Nylander, 2004) or jModelTest (Darriba *et al.*, 2012).

15.7.1 jModelTest: A tool to select the best-fit model of nucleotide substitution

jModelTest (http://code.google.com/p/jmodeltest2/) is a tool to carry out statistical selection of best-fit models of nucleotide substitution evolution that best fits the data and to use in constructing phylogenetic trees in PAUP* or MrBayes. It implements five different model selection strategies: hierarchical and dynamical likelihood ratio tests (hLRT and dLRT), Akaike and Bayesian information criteria (AIC and BIC), and a decision theory method. The jModelTest program is described by Posada (2008) and Darriba *et al.* (2012).

- Double click on jModelTest.jar to open it.
- Go **File > Load DNA alignment** and open the data set file (Fig. 15.12).
- Click on **Analysis > Compute likelihood.** A dialog box will appear that allows you to specify a number of likelihood settings, including the number of substitution schemes to be tested. Click on **Compute Likelihood** to start the analysis.
- When Likelihood score calculation is completed, click on **Analysis** and select either **Do AIC calculations** or **Do BIC calculations**. In the setting window, check a box for **Write PAUP* block** and click on **Do AIC calculation**.
- The best-fit model is displayed with PAUP* block, which can be added to the PAUP* input file. The results can be saved with **Ctrl+S**.

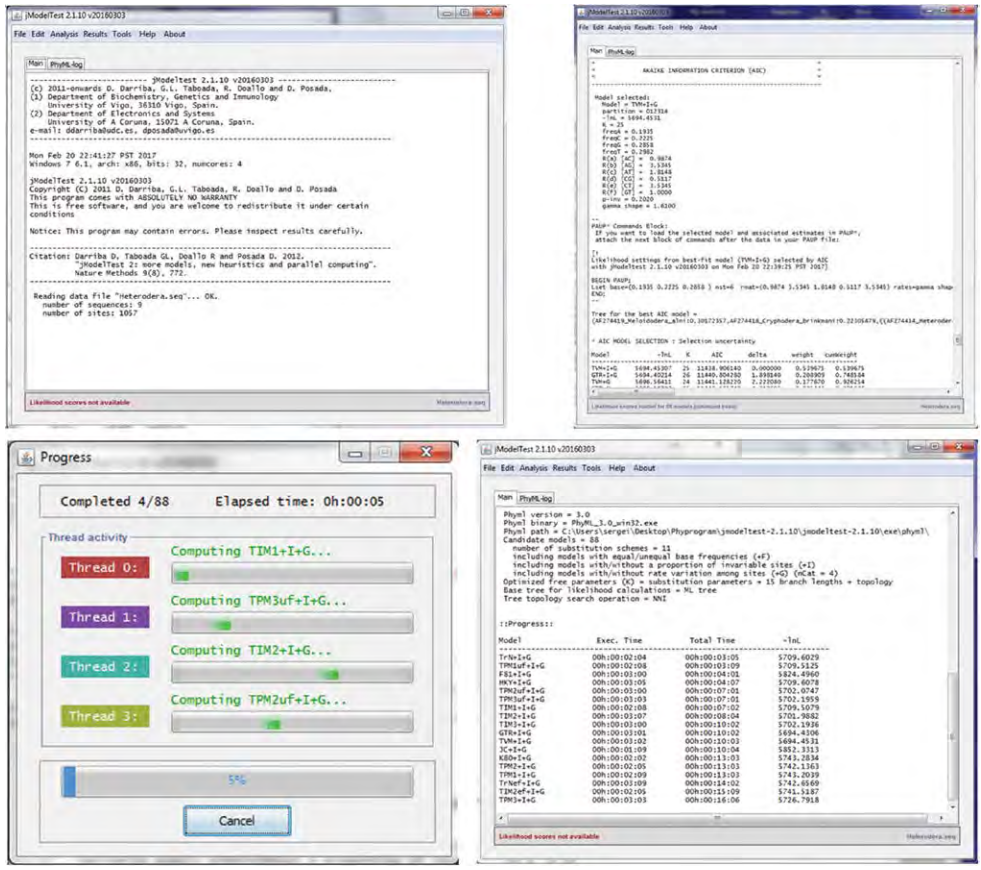

Fig. 15.12. jModelTest windows.

The widely used General Time Reversible (GTR) model has six substitution types (**lset nst=6**), one for each pair of nucleotides and it is the most complex model. In addition to models describing the rates of change from one nucleotide to another, there are models to describe rate variation among sites in a sequence. The following are the two most commonly used models: (i) gamma distribution (G) or gamma distributed rate variation among sites proportion of invariable sites; and (ii) (I) or extent of static, unchanging sites in a dataset.

15.8 Phylogenetic Analysis with PAUP*

Once the sequences are aligned, there are several methods of phylogenetic analysis that can be implemented. The main methods include minimum evolution, parsimony and likelihood methods, and Bayesian inference.

Minimum evolution (distance) calculates a measure of the distance between each pair of species and then finds a tree that predicts the observed set of distances as closely as possible. A topology showing the smallest value of the sum of all branches is chosen as an estimate of the correct tree.

Maximum parsimony (MP) is a character-based method that builds a phylogenetic tree by minimizing the total tree length. It searches for the minimum number of evolutionary steps required for a given set of data.

Maximum likelihood (ML) is a statistical method for reconstructing trees. It requires three elements: (i) sequence alignment; (ii) tree topologies; and (iii) model of character evolution. ML operates by trying to maximize the likelihood value, and the tree with the highest likelihood value is considered the best tree.

These three approaches can be performed using PAUP* software (Swofford, 2003) that is available from Sinauer Associates Inc. Publishers, Sunderland, Massachusetts, USA. PAUP* (Phylogenetic Analysis Using

Parsimony* and other methods) (http://paup.phylosolutions.com) performs phylogenetic analyses using parsimony, maximum likelihood and distance methods. The program features an extensive selection of analysis options and model choices, and accommodates DNA, RNA, protein and general data types.

The Windows versions of PAUP* require data and commands, which should be typed in NEXUS format. PAUP* is run by entering the command blocks. The PAUP* manual with command explanation is given in the PDF documentation in the PAUP* folder: http://paup.phylosolutions.com/tutorials/quick-start/ The PDF command summary can be found in the **Quick Start tutorial and PAUP* FAQ: Answers** pages.

NEXUS data files always begin with the characters #nexus but are otherwise organized into major units known as blocks. The necessary commands can be put in a PAUP* block in the original nexus file after **Assumption block** in any text editor or in PAUP* using **Edit File Open Mode**. Each command begins with a command-name and ends with a semicolon. NEXUS files can contain text comments surrounded by square brackets. The following sections present several examples of the command blocks for distance, parsimony, and likelihood analyses.

15.8.1 Distance method

A. Command block for reconstruction of neighbour-joining tree:

```
begin paup; [start PAUP running]
log file = NJ.log; [command starts a log file]
set criterion = distance; [command defines the optimality criterion]
outgroup 8; [command specifies that the resulting trees should be rooted to given taxon]
bootstrap nreps = 1000 search = nj; [commands specify bootstrap number and method of search]
nj; [command calculates a tree using the neighbor-joining method]
showtrees; [command to request to display one or more trees]
savetrees file = NJ.tre brlens = yes root = yes; [commands save the best rooted tree
  found during the search with branch length information in a file]
log stop; [command stops the logging of output]
end; [stop PAUP running]
```

B. Command block for calculation of nucleotide differences

```
begin paup;
log file = distance.log;
dset distance = mean; [command gives mean number of pairwise character differences,
  adjusted for missing data]
BaseFreq; [command shows base frequencies for each taxon]
showdist; [command shows output a matrix of 'distances' between taxa in a PAUP window]
log stop;
end;
```

15.8.2 Maximum parsimony

A. Command block for reconstruction of maximum parsimony trees

```
begin paup;
log file = MP.log;
set increase = auto; [setting automatically be increased by a number of trees equal
  to the default number 100, could be changed into 'no', if search takes long time]
set autoclose; [closes the status window automatically]
set criterion = parsimony;
outgroup 8;
bootstrap nreps = 1000 search = heuristic;
hsearch nreps = 10 addseq=random; [search for optimal trees using heuristic algorithm
  with 10 replicates using random-addition-sequence replications to be performed]
showtrees;
describetrees /apolist = yes; [command produces a depiction of the tree and set a
  list of the apomorphic characters is displayed]
```

```
savetrees file = MP.tre brlens = yes root = yes;
log stop;
end;
```

B. Command block for obtaining a strict consensus tree

```
begin paup;
log file = conMP.log;
gettrees file = MP.tre; [command to load trees into memory from a file]
outgroup 8;
contree / root = outgroup; [root a strict consensus tree]
showtrees;
contree / treefile = conMP.tre; [save strict consensus file]
log stop;
end;
```

15.8.3 Maximum likelihood

A. Command block for reconstruction of maximum likelihood trees

```
begin paup;
log file = ML.log;
set autoclose;
set criterion = likelihood;
Lset base=(0.1943 0.2232 0.2846) nst=6 rmat=(0.9577 3.5283 1.7964 0.5168 3.5283) rates=gamma
  shape=0.7150 ncat=4 pinvar=0; [model parameters obtained from jModelTest results]
bootstrap nreps = 100 search = faststep; [bootstrap with tree searches in each replication
  are performed using one random-sequence-addition replication and no branch swapping]
hsearch;
savetrees file = ML.tre brlens = yes root = yes;
log stop;
end;
```

- Double click on PAUP icon and run the program. Change **File Open Mode** to **Edit**. Select the executable file and click on **Open** (Fig. 15.13).
- Insert command block or blocks after assumption block in the file.

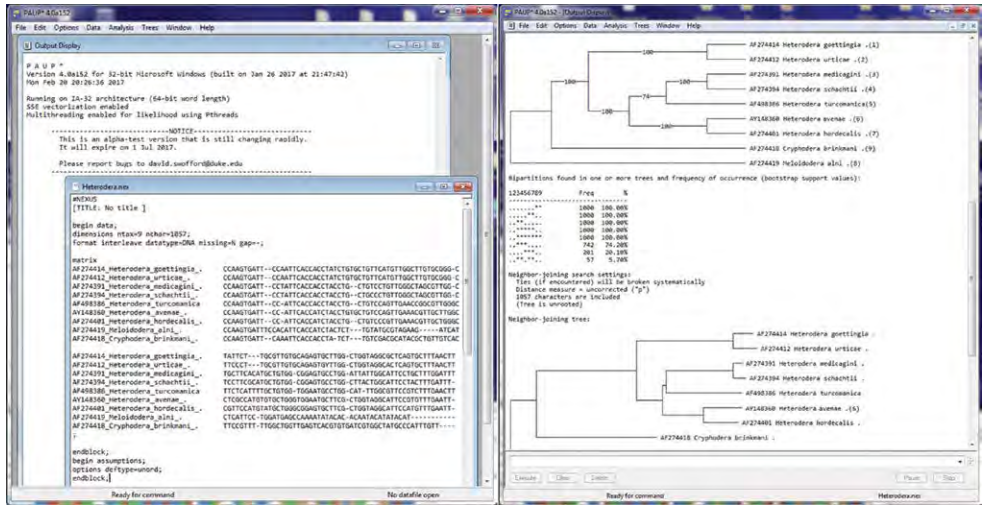

Fig. 15.13. PAUP* with data matrix and outputs.

- Open **File** and then select **Execute** 'file.extension'.
- PAUP performs analysis.

PAUP generates two files: (i) a file with extension.**log** containing information on search, resulted bootstrap tree and table and optimal tree, which can be opened in any text Editor program; and (ii) a file with extension.tre with resulting tree, which can be visualized with the TreeView.

15.9 Phylogenetic Analysis with MrBayes

MrBayes is a program for the Bayesian estimation of phylogeny (http://mrbayes.scs.fsu.edu/). Bayesian inference (BI) of phylogeny is based upon a quantity called the posterior probability distribution of trees, which is the probability of a tree conditioned on the observations. The conditioning is accomplished using Bayes's theorem. MrBayes uses a simulation technique called Markov chain Monte Carlo (or MCMC) to approximate the posterior probabilities of trees (Ronquist and Huelsenbeck, 2003; Ronquist *et al*., 2012).

- Open your nexus format file in any text editor program.
- Type the following command block with appropriate model (model GTR+G+I is used as an example) after the data block.
  Command block for reconstruction of BI trees:

```
begin mrbayes; [command to start MrBayes]
log start filename = BIfile.log; [command to create the log file under certain name]
outgroup 8; [command specifies that the resulting trees should be rooted to given taxon]
lset nst=6 rates=invgamma; [commands to set parameters for the model]
showmodel; [command to show model settings]mcmc ngen=1000000 printfreq=100
  samplefreq=100 nchains=4 savebrlens=yes filename=BItree; [commands to run the Markov
  chain with 1000000 generations, print the results to the screen every 100 generations,
  record the current tree and parameters values to files every 100 generations, run 4
  independent chains, save the trees with branch lengths in the tree file]
sumt filename=BItree burnin=1000 contype=halfcompat; [commands to summarize the saved
  trees, discard the first 1000 trees or 10% of total saved trees and write a majority
  rule consensus tree in BItree.con]
sump filename=BItree burnin=1000; [commands to summarize statistics for trees sampled
  during analysis]
log stop; [command to quit recording in the log file]
end;
```

  Command to set models: JC - nst = 1; K2, HKY, T3P, TN93 - nst = 2; GTR - nst = 6 and rates: Uniform - rates = equal; Gamma Distributed (G) - rates = gamma; Invariant Sites (I) - rates = propinv; G + I - rates = invgamma

- **Save** the file. Copy MrBayes in the same directory.
- Double click on MrBayes icon and run program. Type the following command: **execute** and after a space type **file name with extension**. Click > **Enter**

The program runs (Fig. 15.14). Each result line shows the generation number at the left, then a series of four numbers, each enclosed in brackets. Those numbers are the log likelihood of the trees in each of the four chain in run 1. The cold chain is enclosed in square brackets. After symbol * – chain is in run 2. The last number in each line of output to the screen is the estimated time until the run ends (Figs 15.15 and 15.16). If the average standard deviation of split frequencies is stabilized for many generations below 0.01, the run can be stopped. The results of BI analysis are saved in several files: BIfile.log, BItree.mcmc, BItree.run1.p, BItree.run2.p, BItree.run1.t, BItree.run2.t, BItree.con.

The BItree.con file can be opened in TreeView to visualize the tree.

Several other software applications can be used to reconstruct phylogenetic relationships: RAxML (https://sco.h-its.org/exelixis/web/software/raxml/) and BEAST (http://tree.bio.ed.ac.uk/software/beast) and MEGA (https://www.megasoftware.net).

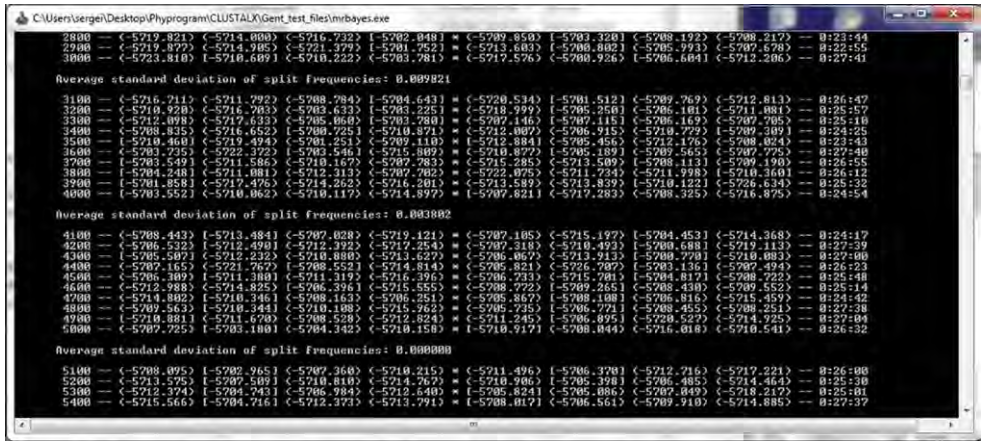

Fig. 15.14. Output while MrBayes is running.

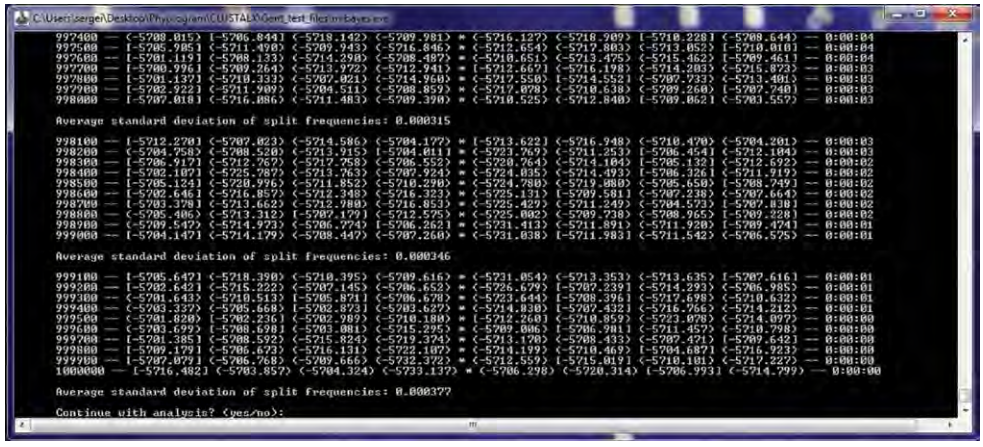

Fig. 15.15. Output of MrBayes run.

15.10 Visualization of Phylogenetic Trees

TreeView is a program for displaying and printing phylogenies (https://code.google.com/archive/p/treeviewx/). TreeView provides a simple way to view the contents of a NEXUS, PHYLIP, Hennig86, Clustal, or other format tree file (Page, 1996). The PAUP* for Windows does not have a graphical interface, hence TreeView allows you to create publication quality trees from PAUP files, either directly, or by generating graphics files for editing by other programs.

- Run TreeView program. Choose **File > Open**. Then select the file in your folder. Click **Open**. The tree appears in TreeView (Fig. 15.17).
- Click on **Phylogram** icon to see the tree with length branches. Choose **Tree > Order > Select Ladderize left > OK.** Outgroup taxa appear in the bottom of the tree.
- If the tree needs to be re-rooted, select **Tree > Define outgroup** and then select Outgroup taxa. Click OK.
- The displayed tree can be saved in a graph format (.emf). Choose **File > Save as graph**, then type file name, select format**. emf** and then click **Save.**

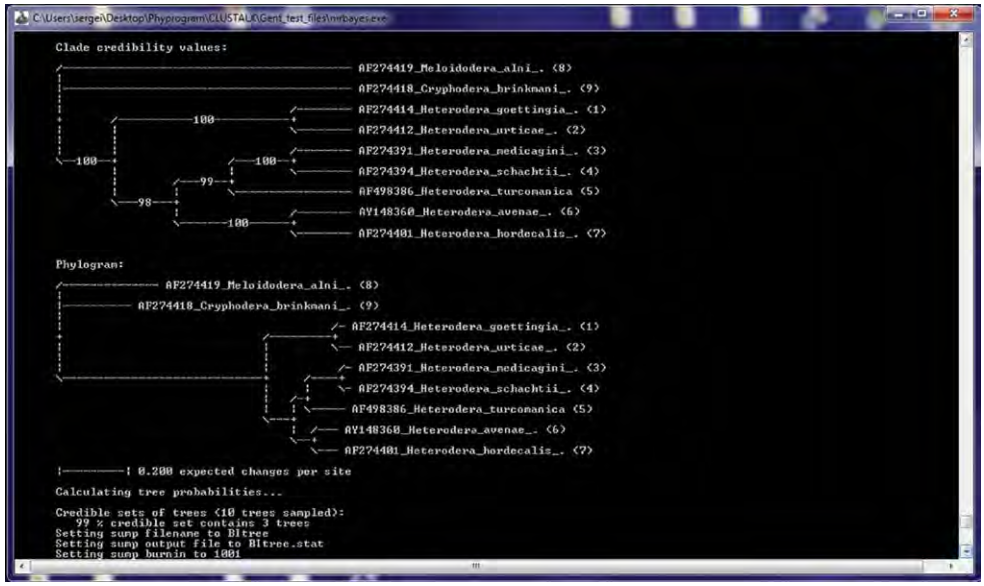

Fig. 15.16. Tree output of MrBayes run.

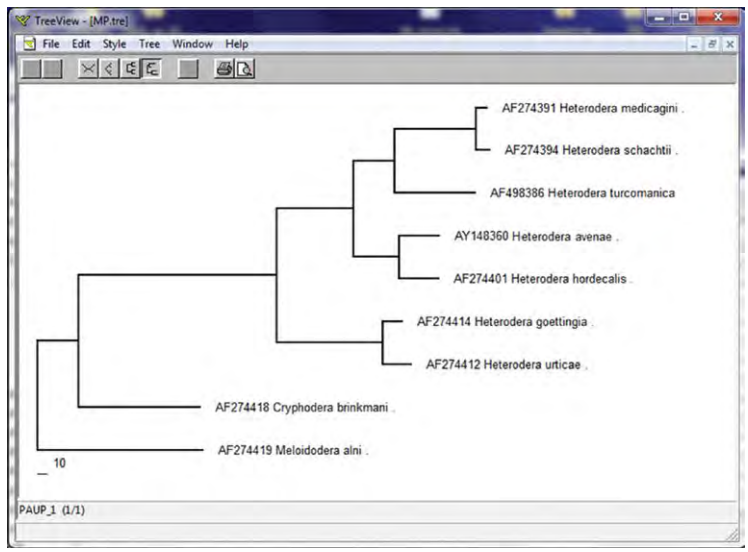

Fig. 15.17. Tree visualized by TreeView.

This file can be opened and edited by Adobe Illustrator, CorelDraw, Inkscape or other Graph Editors.

FigTree is another graphical viewer of phylogenetic trees and a program for producing publication-ready figures (http://tree.bio.ed.ac.uk/software/figtree/). In particular, it is designed to display summarized and annotated trees produced by BEAST.

Inkscape (https://inkscape.org) is professional quality vector graphics software that runs on Windows, Mac and Linux. It is used for creating a wide variety of graphics and can be used for final preparation of a phylogenetic tree for a publication. The program can be freely downloaded from the website.

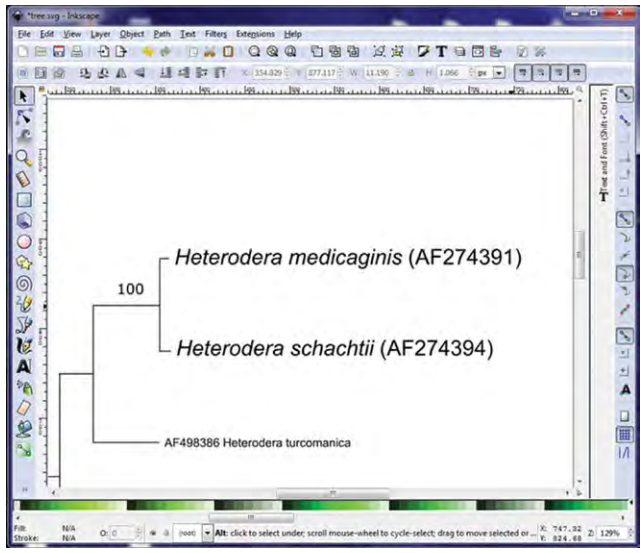

Fig. 15.18. Inkscape with a tree.

The phylogenetic tree should contain clear terminal labels with species names and GenBank accession numbers and numbers with bootstrap or posterior probability values placed on appropriate branches (Fig. 15.18).

15.11 Sequence Submission

The final and important step of a phylogenetic study is the submission of new sequences in public databases. Once sequences are submitted and accession numbers are assigned, these numbers must be included in a publication and published tree. New sequences should be submitted in one of the public database: GenBank, a comprehensive public database of nucleotide sequences and supporting bibliographical and biological annotation built and distributed by NCBI (Benson *et al*., 2010); EMBL (the European Molecular Biology Laboratory Nucleotide Sequence Database in Europe); or DDBJ (DNA Data Bank of Japan). Daily data exchange within these databases ensures worldwide coverage.

There are some options for submitting data to GenBank:

- **BankIt** (https://www.ncbi.nlm.nih.gov/WebSub/), a WWW-based submission tool with wizards to guide the submission process; or
- **Submission Portal** (https://submit.ncbi.nlm.nih.gov), a unified system for multiple submission types. Currently only ribosomal RNA (rRNA), rRNA-ITS, Influenza or Norovirus sequences can be submitted with the GenBank component of this tool.

15.12 References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* 215, 403–410. DOI: 10.1016/S0022-2836(05)80360-2

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. and Sayers, E.W. (2010) GenBank. *Nucleic Acids Research* 38, D46–D51. DOI: 10.1093/nar/gkp1024

Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* 17, 540–552. DOI: 10.1093/oxfordjournals.molbev.a026334

Darriba, D., Taboada, G.L., Doallo, R. and Posada, D. (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* 9, 772. DOI: 10.1038/nmeth.2109

Hall, B.G. (2017) *Phylogenetic Trees Made Easy*. 5th edn. Oxford University Press, Oxford, UK.
Huang, X. and Madan, A. (1999) CAP3: A DNA sequence assembly program. *Genome Research* 9, 868–877. DOI: 10.1101/gr.9.9.868
Larkin, M.A., Blackshields, G., Brown, N.P. *et al.* (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948. DOI: 10.1093/bioinformatics/btm404
Lemey, P. Salemi, M. and Vandmme, A.-M. (eds) (2009) *The Phylogenetic Handbook: A Practical Approach to Phylogenetic Analysis and Hypothesis Testing*. 2nd edn. Cambridge University Press, Cambridge, UK. DOI: 10.1111/j.1541-0420.2010.01388.x
Nicholas, K.B., Nicholas Jr, H.B. and Deerfield II, D.W. (1997) GeneDoc: analysis and visualization of genetic variation. *EMBNEW News* 4, 14.
Nylander, J.A.A. (2004) *MrModeltest Version 2. Program distributed by the author*. Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.
Page, R.D.M. (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12, 357–358.
Posada, D. (2008) jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* 25, 1253–1256. DOI: 10.1093/molbev/msn083
Posada, D. and Crandall, K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817–818. DOI: 10.1093/bioinformatics/14.9.817
Ronquist, F. and Huelsenbeck. J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574. DOI: 10.1093/bioinformatics/btg180
Ronquist, F., Teslenko, M., van der Mark, P. *et al.* (2012) MrBayes3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61, 532–542. DOI: 10.1093/sysbio/sys029
Swofford, D.L. (2003) PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b 10. Sinauer Associates, Sunderland, Massachusetts, USA.
Talavera, G. and Castresana, J. (2007) Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* 56, 564–577. DOI: 10.1080/10635150701472164
Wheeler, D. and Bhagwat, M (2007) Chapter 9. BLAST QuickStart In: Bergman N.H. (ed.). *Methods in Molecular Biology, Vol. 395: Comparative Genomics*. Humana Press Inc., Totowa, New Jersey, USA, pp. 149–175.
Yang, Z. and Rannala, B. (2012) Molecular phylogenetics: principles and practice. *Nature Review: Genetics* 13, 303–314. DOI: 10.1038/nrg3186

Index

Page numbers in *italics* refer to illustrations

CABI – who we are and what we do

This book is published by **CABI**, an international not-for-profit organisation that improves people's lives worldwide by providing information and applying scientific expertise to solve problems in agriculture and the environment.

CABI is also a global publisher producing key scientific publications, including world renowned databases, as well as compendia, books, ebooks and full text electronic resources. We publish content in a wide range of subject areas including: agriculture and crop science / animal and veterinary sciences / ecology and conservation / environmental science / horticulture and plant sciences / human health, food science and nutrition / international development / leisure and tourism.

The profits from CABI's publishing activities enable us to work with farming communities around the world, supporting them as they battle with poor soil, invasive species and pests and diseases, to improve their livelihoods and help provide food for an ever growing population.

CABI is an international intergovernmental organisation, and we gratefully acknowledge the core financial support from our member countries (and lead agencies) including:

UKaid from the British people

Ministry of Agriculture People's Republic of China

Australian Government Australian Centre for International Agricultural Research

Agriculture and Agri-Food Canada

Ministry of Foreign Affairs of the Netherlands

Schweizerische Eidgenossenschaft Confédération suisse Confederazione Svizzera Confederaziun svizra

Swiss Agency for Development and Cooperation SDC

Discover more

To read more about CABI's work, please visit: **www.cabi.org**

Browse our books at: **www.cabi.org/bookshop**,
or explore our online products at: **www.cabi.org/publishing-products**

Interested in writing for CABI? Find our author guidelines here:
www.cabi.org/publishing-products/information-for-authors/

Techniques for Work with Plant and Soil Nematodes

Edited by

Roland N. Perry, David J. Hunt and Sergei A. Subbotin

Plant-parasitic and free-living nematodes are increasingly important in relation to food security, quarantine measures, ecology (including pollution studies) and research on host-parasite interactions. Being mostly microscopic, nematodes are challenging organisms for research. *Techniques for Work with Plant and Soil Nematodes* introduces the basic techniques for laboratory and field work with plant-parasitic and free-living soil-dwelling nematodes.

Written by an international team of experts, this book is extensively illustrated, and addresses both fundamental traditional techniques and new methodologies. The book covers areas that have become more widespread over recent years, such as techniques used in diagnostic laboratories, including computerized methods to count and identify nematodes. Information on physiological assays, electron microscopy techniques and basic information on current molecular methodologies and their various applications is also included.

This book is an essential resource for students of nematology and parasitology, academic researchers, diagnostic laboratories, and quarantine and advisory service personnel. It provides a much-needed methodology standard for anyone involved in work on plant and soil nematodes.

Cover image: *Ditylenchus dipsaci* adult female. Photograph, courtesy of Jon Eisenback, Department of Plant Pathology, Virginia Tech, USA.