Fungicides in Practice

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Fungicides in Practice

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Preface

The motivation for writing *Fungicides in Practice* is to provide an up-to-date guide to the science and practice of disease control based on fungicides in horticulture and broad-acre agriculture. The book is aimed at students of agriculture and agronomy with an interest in disease control. It also serves as a primer for the broad range of people – chemists, biochemists, molecular biologists, microbiologists – who work or seek to work in the fungicide industry. We also hope that the book will be useful to the direct users of fungicides – farmers and growers of all sorts – plus their advisors.

The book owes a debt to the two editions of *Fungicides in Crop Protection* (Oliver, R.P. and Hewitt, G.H., 2014; and Hewitt, G.H., 1998). Some of the structure is retained and the content substantially updated. New actives have been brought to market. Much progress has been made in the science of resistance evolution and management. Regulatory issues have led to the withdrawal of many previously useful actives. GM disease resistance gene deployment remains stalled but new methods of genome editing promise to move the field forward. RNA-based fungicides are also on the horizon.

A new chapter on disease control using crop protection products in Organic agriculture is added. This covers basic substances (copper, sulfur) in some detail plus botanicals and biological control agents. Many of these products are also used in conventional agriculture.

Feedback from the 2nd edition led us to add four new chapters which describe fungicide formulation, mobility, application to the crop and tactics in use, in addition to a chapter on the experimental design of fungicide trials and their analysis. These chapters have been led by Janna Beckerman.

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I would like to thank my colleagues in industry, agriculture and universities for discussion and comments. This volume has been prepared with the help of many people in the fungicide industry who have provided me with insight into the world of fungicides over the last 20 years. These include the late Andy Leadbeater, Derek Hollomon, Craig White, Craig Ruchs, Craig Pensini, Jenny Davidson, Doug Wilson, Fran Lopez-Ruiz, Frank van den Bosch, Gavin Heard, Geoff Robertson, Gerd Stammler, Hans Cools, Kithsiri Jayasena, Ken McKee, Kevin Bodnaruk, Lise Nistrup Jørgensen, Michael Csukai, Naomi Pain, Neil Paveley, Nick Poole, Peter Hobbelen, Rick Horbury, Scott Paton, John Lucas, Bart Fraaije, Andy Corran, James Brown, Susan Knight, Melvin Bolton and many others. The book was largely written during the COVID-19 pandemic. I am grateful to my office mate, Professor Mary Oliver, for support.

Richard Oliver

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I would like to thank the many growers whose questions shaped my research, particularly Jean-Marc Versolato (Bailey Nursery) and Eric Nordlie (Bachman Greenhouses), who, early in my career, focused my attention to fungicides and practical disease management. I would also like that thank all the Indiana apple growers, but especially Sarah Brown (AppleWorks Orchard), Dave Byers (ApplAcres), the Roney family (Tuttle's Orchard), David Doud (Doud's Countyline Orchard) and Brian Garwood (Garwood Orchard). It has been my honour to work with all of you. I would also like to thank my friends Richard Latin, Darcy Telenko (Purdue University) and Kiersten Wise (formerly Purdue University; University of Kentucky); George Sundin (Michigan State University), Kerik Cox, David Rosenberger and Margery Daughtrey (Cornell University – Geneva, Hudson Valley, Long Island Campuses); Renee Keese, Jennifer Bergh-Browning and Kathie Kalmowitz (BASF); John Phillips and Stuart Brennemen (Nutrien Ag Solutions); Frank Wong and Aaron Palmateer (Bayer); and Eric Tedford and the late Andy Leadbeater (Syngenta). Thank you all for the wonderful conversations on fungicides and plant disease, particularly when we had those conversations over food and 'craft' cocktails. Lastly, I need to thank my daughter, who had to sit through so many of these conversations at home and at meetings, from a young age, and somehow managed to be the phenomenal person she is despite my parenting.

Janna Beckerman

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1

Introduction

Fungicides can be defined as products of natural or synthetic origin, which act to protect plants against invasion by fungi and/or to eradicate established fungal infection. They include chemicals which have direct activity against fungi as well as ones that stimulate the existing defences of the plant. Conventional fungicides are chemicals and include both natural and synthetic products, but we can also consider living organisms as a distinct class of fungicides, better known as biological control agents (BCAs).

Alongside herbicides, insecticides and plant growth regulators, fungicides form the battery of agrochemicals (also known as pesticides) that are available to protect crops and maintain their yield potential, measured as the quantity or quality of produce. Diseases of crops are caused by a diverse range of organisms that include the true fungi (e.g. *Ascomycota* and *Basidiomycota*), the fungal-like but unrelated *Oomycota* (e.g. *Phytophthora* and *Pythium*), the club-root pathogen *Plasmodiophora*, as well as various bacterial, viral and nematode species. The term fungicide is conventionally taken to mean compounds that control organisms that look like fungi. This includes the true fungi, *Cercozoa* and the *Oomycota*. It does not include chemicals that control bacteria (these compounds are conventionally called antibiotics), viruses (mainly controlled by insecticides) or nematodes (mainly controlled by genetic and cultural methods).

Pesticide use dates from the 18th century and became almost ubiquitous by the middle of the 20th. Several compelling factors have ensured their widespread use and fuelled the growth of the pesticide discovery and supply industries. These include an increasing world population, with higher incomes meaning that food demand has risen steeply. Furthermore, there are direct and clear benefits both to the grower, such as higher and more consistent yields, lower labour costs and greater profit, and to the consumer, such as consistency of food quality, increased variety of produce and lower prices.

Population Growth and Food Production

For most of recorded history, the global population growth rate has been below 0.2% per annum. However, the early 19th century witnessed the beginning of an accelerating advance in the control of human diseases that initiated a dramatic reduction in mortality rates, especially of infants. High birth rates resulted in a rapid increase in population growth which, in the industrialized nations, levelled out after the 1960s. Greater food security in parts of Asia and Africa means that their populations are still expanding rapidly. None the less, nearly one in nine people (or 820 million people) globally were hungry or

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undernourished in 2020, with 132 million of them living with acute hunger that approached starvation (McCarthy and Sánchez, 2020).

The world population is currently estimated at 7.8 billion, having increased from 6 billion just 20 years ago. Conservative estimates predict a world population of 10 billion by 2060. An increasing proportion of the world's population is demanding a diet that is higher in dairy and meat produce. The animals are increasingly fed on grain and on silage or hay made from land suitable for growing crops for direct human use. The area of land available to grow all these crops is under threat from urbanization, pollution and climate change. We, as members of the community, can take part in debates about limiting the world's population, reducing the degree of pollution, limiting the consumption of animal products and non-productive use of land. None the less, there can be no escaping the conclusion that there is an unequivocal and urgent need to produce more food that is nutritious and safe on less land, using less water and fertilizers.

Historically, the world's increasing demand for food has been met largely through an expansion of the area under cropping and by improvements in the food distribution network. The increased food needs of Western Europe in the 19th century, for example, were supplied by the expansion of production in the Americas and Australasia. The 20th century introduced a technological revolution into agriculture which has made possible a rapid rate of growth of food production to feed a historically unprecedented rapid growth of world population. Central to the growth in food production was the development of artificial fertilizers and high-yielding crop varieties – the Green Revolution (Evenson and Gollin, 2003). The high yields, monocultures and fertilizer inputs increased disease levels. This both increased the need for fungicides and justified their costs.

Agriculture makes a significant impact on global warming (Berry *et al.*, 2010). About a seventh of all greenhouse gas (GHG) emissions can be ascribed to agriculture. These include direct use of fossil fuels for transport and tillage, indirect use of fossil fuels for nitrogen fertilizer production, and GHG emission due to soil microbe release of methane and nitrogen oxides. Much of this is due to emission by ruminant animals. It is therefore possible to quantify food production not just on a tonne per hectare basis but also on a tonne per GHG emission basis. Such studies consistently show that the disease control and green leaf area duration promoted by appropriate use of fungicides maximizes food production both per hectare and per GHG equivalent (Berry *et al.*, 2008). For example, in the case of UK barley production, efficient control of foliar disease by fungicides decreased GHG emissions by 29–60 kg CO_2 eq./t in UK winter barley (Hughes *et al.*, 2011). There is a strong argument for appropriate use of fungicides to combat climate change.

The impact of disease on crop production

It is notoriously difficult to estimate the scale of losses caused by disease. Savary *et al.* (2019) surveyed experts and derived estimates of the losses due to pathogens and pests in five major crops. They documented losses associated with 137 pathogens and pests (mainly insects and nematodes) in wheat, rice, maize, potato and soybean worldwide. The yield loss average estimates were 21.5% for wheat, 30.0% for rice, 22.5% for maize, 17.2% for potato and 21.4% for soybean. However, some areas – particularly in regions with rapidly expanding human populations – reported losses of more than 40%. The great majority of the losses are caused by fungi and oomycetes. These numbers show that all the means to control disease, genetics, cultural methods as well as fungicides, need to be used in concert to achieve adequate food production in a sustainable manner.

Agricultural Technology and the Impact of Fungicide Use

Crop production is a process governed by a series of limiting factors which interrelate. These include crop variety (i.e. the varying degree of genetic disease resistance), nutrition, water supply, soil quality and crop management (pest, weed and disease control, cultivation). Each factor may assume a dominant, yield-limiting role, depending upon the crop, husbandry practices and the region. For example, water availability is the major factor governing plant distribution

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and is often the determining factor in yield production. Historically, the combined action of improvements in irrigation and the introduction of new varieties with higher genetic potential for yield resulted in dramatic yield increases. Later, the use of fertilizers relieved the limitations to yield dictated by nutrient deficiency and allowed the inherent yield capacity of the crop to be realized to a point that was limited by photosynthetic light interception, weed populations, insect infestation and disease. In the 20th century, intensive breeding programmes have further improved the genetic potential for yield in many crops and their capability to respond to other inputs such as fertilizers and agrochemicals.

One of the consequences of increased fertilizer use is more frequent and damaging attacks by fungi, and in intensively grown crops their control is a significant factor in yield determination. The improved control of diseases has permitted an even greater use of fertilizer and further increases in yield. Many authorities agree that intensive use of good-quality agricultural land for food production is the best way to feed the world and to free up poorer areas for biodiversity preservation.

Since the 1940s, the search for new fungicides has intensified and the total value of the crop protection business, as fungicide sales, stood at $15.1 billion in 2017 compared with $13 billion in 2013 and $6 billion in 1995 (all monetary values are US$ unless stated otherwise). The economics of pesticide use vary from crop to crop, between targets and according to the levels of weed, insect or disease infestation. Studies in Australia document the gain of AUS$8 for every AUS$1 spent on fungicides (Murray and Brennan, 2009, 2010). This figure is driven by the sharp reductions in the cost to farmers for some fungicides in the last 15–20 years. The cost of off-patent fungicides has fallen to less than AUS$5/ha and so disease gains need only be small to justify the costs. The value gained from the use of small amounts of fungicide to control seed-borne diseases is also very large. More modest but still significant gains are obtained when controlling foliar diseases. The use of cereal fungicides in Western Europe accounts for an extra 2–3 Mt of grain annually, equal to $400–600 million. In some cases, the benefit gained through fungicide use is more critical because certain crops cannot be cultivated in the absence of disease control. By the late 1800s coffee rust epidemics were a serious and frequent problem in India, Sri Lanka and Africa. Eventually, production levels became uneconomic and stimulated a change in cropping from coffee to tea. The recovery of the coffee industry was, and remains, totally dependent on the use of fungicides.

The impact of fungicide use on wheat in the UK is illustrated in Fig. 1.1. The average yield of wheat in the UK increased from about 4 to 8 t/ha from 1974 to 2000. Since then, average yields have stagnated, but maximum yields have continued to increase. One farmer reported a yield of 14.3 t/ha on a commercial crop in 2013. During this period, methyl benzimidazole carbamate (MBC) fungicides were introduced from 1972, then demethylation inhibitor (DMI) fungicides were introduced from 1978, quinone outside inhibitor (QoI) fungicides were introduced in 1998, and second-generation succinate dehydrogenase inhibitor (SDHI) fungicides came in after 2003. The uptake of foliar fungicides was dramatic, rising from zero in 1972 to more than 90% by 1986. Since 2000 the number of fungicide applications on an average field has increased, even though the total weight has declined. This trend reflects the need to protect the crop during critical growth phases as well as the need to use different actives to combat different pathogens and fungicide resistance.

A 2005 survey by CropLife America of US crops estimated the cost of fungicide use on different types of crops and the extra yield that was obtained from better disease control. Across all sectors the increased yield amounted to $12.8 billion on an expenditure of $880 million; that is, a ratio of 14.6:1. There were substantial variations in the benefit:cost ratio. Perennial crops like grapevines and apples had ratios of about 20 and the value for potatoes was 11. The lowest ratio was 1.8 for wheat reflecting the modest yield obtained in North America.

Wheat yields are generally much higher in Europe and a key focus for the fungicide industry is the control of septoria tritici blotch. Yields in the UK, Germany and France are typically 7–10 t/ha and the total production is about 74 Mt or close to 10% of global production. Despite a highly intensive plant breeding effort, an educated and well-equipped farming community and the first use of new fungicides, losses to septoria tritici blotch are stubbornly high at 5–10%.

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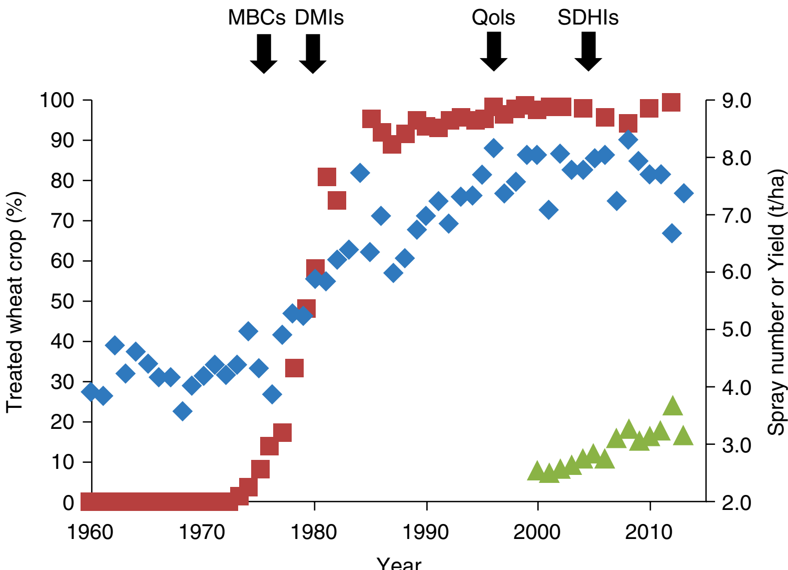

Fig. 1.1. Wheat yields and fungicide use in the UK, 1960 to 2013. Wheat yields (◆); percentage of crops sprayed with fungicides (■); average number of sprays per season (▲); and introduction of main fungicide groups (arrowed; MBCs, methyl benzimidazole carbamates; DMIs, demethylation inhibitors; QoIs, quinone outside inhibitors; SDHIs, succinate dehydrogenase inhibitors). (From Lucas *et al*., 2015, with permission from Professor John Lucas.)

Fungicides are applied at a cost of $1 billion/year, but they result in an increased yield averaging 2.5 t/ha. This equates to a return on investment of about 5 to 1 (Fones and Gurr, 2015). The economic impact of fungicide use on wheat in the UK, Germany and France was estimated as $15 billion/year.

Detailed studies of disease losses and fungicide use have been made in Australia for wheat and barley (Murray and Brennan, 2009, 2010). Australia has a generally low rainfall and poor soils, giving average cereal yields in the range of 1–2 t/ha. These are conditions in which disease levels would be expected to be low by world standards. It is sobering that even under these close-to-ideal conditions, highly researched pathogens still cause up to 30% losses in competently farmed crops (Table 1.1). Table 1.2 details the absolute actual loss in Australian dollars in comparison to the loss expected if no control methods (genetics, cultural or fungicide) were applied. The difference between the potential loss and the actual has been apportioned to each of the major control methods. Fungicides have a very significant role in protecting yield. This

Table 1.1. Estimates of losses due to disease in major crops in Australia. (Modified from Murray and Brennan, 2009, 2010; Murray, 2012.)

Crop	% yield lost to diseases
Wheat	18.0
Barley	13.5
Field pea	29.6

varies between disease, crop, variety and season, but overall the annual AUS$250 million expenditure on fungicides in Australia generates a return of AUS$2000 million.

The History of Fungicide Use

The devastating social effects of plant disease are a common feature of history, extending into Biblical times and beyond with references to 'blasting and mildew' in the books of Deuteronomy and Amos (Large, 1940/2003; Agrios, 2005; Money, 2006). Wheat rusts were known at least from Roman times and were considered so

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Table 1.2. Breakdown of losses to disease and gains to genetic, cultural and chemical disease control in selected grain crop diseases in Australia; all figures are in AUS$ million. The 'potential loss' is the loss incurred if no control measures were in place; the 'actual loss' is the current estimate. The difference between potential and actual is assigned to either genetic control, cultural practices or fungicide control. It is clear even in low-input, sustainable agriculture situations like Australia that fungicides contribute heavily to disease control. (From Murray and Brennan, 2009, 2010.)

Disease	Potential loss	Actual loss	Genetic control	Cultural control	Fungicide control
Tan spot	676	212	200	155	108
Stripe rust	868	127	431	78	359
Septoria nodorum	230	108	36	51	35
Barley mildew	103	39	10	3	52

important that their occurrence was attributed to divine action. Regular festivals to appease the gods Robigus and Robigo were held in the hope that cereal rust disease could be prevented. However, the gods were clearly not to be trusted and some rudimentary chemical disease control was also practised, the therapeutic but mysterious nature of sulfur being passed down from the ancient Greeks.

Other than crop failure, fungal disease can have a dramatic and direct effect upon human welfare. In 943, a European chronicler described the 'wailing and writhing' of men in the street suffering from a disease which came to be known as 'St Anthony's fire', named after the behaviour of people who, in hope of a cure, visited the shrine of St Anthony in France. The cause is now known to be rye grain contaminated with the alkaloids present in the ergot fungus *Claviceps purpurea*.

By 1750, cereal diseases had attained such a significant economic status in Europe that the French Academy of Arts and Sciences volunteered a prize for the best treatise describing the cause and control of wheat bunt. The solution was not forthcoming and 10 years later up to half of the French wheat crop failed because of bunt and smut (*Ustilaginomycetes*) diseases. Mathieu Tillet eventually characterized the causal organism of wheat bunt, which carries his name, *Tilletia tritici*, and went on to describe the life cycle of the fungus. Of equal importance was the work, based on a series of field experiments, which examined the efficacy of various treatments against *T. tritici*. It was demonstrated that crops treated with various materials mixed with lime or putrefied urine could be maintained relatively free from bunt disease and these treatments came to be of major economic importance in France.

The catalogue of incidents of fungal disease during the 19th century is extensive (Table 1.3). However, the greatest social impact of plant disease was surely the Irish potato famine triggered by potato late blight, *Phytophthora infestans*. In the years following 1845, over 1 million people died and 2 million more were forced to emigrate due to malnutrition, mainly to North America. The population of Ireland is still well below the level achieved prior to the outbreak.

Plant disease was a critical factor in the survival of some commercial industries. The wine industry, for example, was under continual attack; first from grape powdery mildew initially observed in England in 1845 and 3 years later in France and the rest of Europe. This period also witnessed the beginnings of fungicide use. Observations by the gardener who first reported grape powdery mildew in England suggested that applications of sulfur could be used to control the disease. His findings were confirmed by Professor Duchartre of the Institut Agronomique, Versailles, but the challenge to produce a product that could be applied easily to an extensive area of vineyards was not successful until 1855, when Bequerel produced a fine form of sulfur that could be used to achieve effective plant coverage.

Similar advances were made in 1885 with Millardet's invention of Bordeaux mixture, copper sulfate and lime, for the control of grape downy mildew. This procedure was also later shown to be effective against late blight in potatoes. Several versions of the treatment were explored but the mixture developed then is still in use today for the control of fungal and oomycete

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Table 1.3. Major outbreaks of fungal disease in the 19th and 20th centuries. (Modified from Oliver and Hewitt, 2014.)

Crop	Pathogen	Year reported	Region
Cereals	*Claviceps purpurea* (ergot)	1816	France
Hops	*Sphaerotheca humuli* (powdery mildew)	1840	England
Potatoes	*Phytophthora infestans* (late blight)	1845	Europe
Vines	*Uncinula necator* (powdery mildew)	1845	England
Vines	*U. necator*	1848	France
Vines	*Plasmopara viticola* (downy mildew)	1865	France
Coffee	*Hemileia vastatrix* (coffee rust)	1869	Sri Lanka
Vines	*Guignardia bidwellii* (black rot)	1880	France
Cereals	*Puccinia* spp. (rusts)	1889	Austria
Cereals	*Puccinia* spp.	1892	Prussia
Cereals	*Puccinia* spp.	1894	USA
Cereals	*Puccinia* spp.	1916	Canada, Denmark, Russia, Argentina, South Africa, India
Maize	*Cochliobolus heterostrophus* (Southern corn leaf blight)	1970	USA

diseases on a wide range of crops. It is particularly important in 'Organic' agriculture as it is one of the few effective treatments that has regulatory approval by most certifiers of this type of production.

The technology developed in France in response to the frequency and severity of crop disease, especially in vines, became the stimulus for other international investigations. This led, in 1886, to a large programme of trials in the USA to evaluate all the leading French fungicides to protect high-value crops. Early examples were black rot of vines caused by *Guignardia bidwellii*, apple scab caused by *Venturia inaequalis*, gooseberry mildew caused by *Sphaerotheca fuliginea* and several vegetable pathogens. This collaboration between the US Department of Agriculture (USDA) and French experts was one of the first to examine the relationship of dose–response, cost of spray per hectare, optimum timing and phytotoxicity.

The cereal rust diseases that had persisted throughout this period of fungicide development evaded similar attempts at control. Farmers attempted to use resistant varieties and early sowing to combat the disease, but any little success was typically short-lived due to what became known as the 'boom and bust cycle' (McIntosh, 2007). Little success was achieved and by the turn of the 19th century, world wheat production was severely limited by rust infection, a situation destined to remain until the advent of systemic fungicides in the mid-1960s. Other crops also suffered from rust diseases. In 1869, coffee rust was reported in what became Sri Lanka and in 10 years reduced average yields by over 50% to 251 kg/ha. The effective destruction of the coffee industry led to investment in a replacement crop, tea. Henceforth, the cultivation of coffee in India and Sri Lanka was totally dependent on the use of fungicides to control rust disease. An excellent and lively introduction to the social history of plant pathology can be found in Money (2006).

The modern chemical industry can be said to date from the accidental synthesis of mauveine, an aniline dye, by Perkins in London in 1856 (Garfield, 2000). The early goals were to produce fabric dyes to replace the expensive and fade-prone natural products. Large research and production facilities were developed notably in the Rhine valley in Germany and Switzerland, where the forerunners of the today's BASF, Bayer and Syngenta were established. Together with companies in the UK and USA, they diversified to produce the myriad synthetic and natural chemical products that underpin all aspects of modern society. The key expertise of these companies was in the synthesis of novel compounds. Initially the number of compounds was small and so they could all be tested for a variety of applications as dyes, preservatives, pharmaceuticals and explosives, as well as agrichemicals.

The use of complex organic chemistry in crop protection began with the introduction of new seed treatments found to be effective for the

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control of wheat bunt. Studies in the pharmaceutical industry developed phenolic compounds made from arsenic and metallic elements such as mercury, copper and tin. The discovery by the Bayer Company of a compound containing mercury and chlorinated phenol, active against wheat bunt, prompted the intensive development of organomercury seed treatments; the first, Uspulam, being introduced in 1915 by Bayer, followed by Ceresan from ICI (1929) and Agrosan G, also from ICI (1933). The efficacy of these products ensured their widespread popularity in the farming community, and they led the cereal seed-treatment market until mercury-based products were banned in the 1970s and 1980s on the grounds of adverse toxicology.

It was not until after the Second World War that the potential of fungicide use in crop protection and the maintenance of yield were realized, and it is generally accepted that this marks the real beginning of crop fungicide technology. The early fungicides business was founded on the control of crop diseases that previously had been unchecked and competition between companies was relatively light. Most of the products that were introduced were in response to clear needs of growers and they created new markets by exploiting latent demand. Later products improved on existing control and were established at the expense of their lesser competitors. This is particularly true of the introduction from the 1960s of fungicides that were able to move within plants and throughout crops, the so-called systemic or mobile materials, which captured a significant part of the market previously held by surface-bound non-systemic (immobile) products such as sulfur and copper-based materials.

Fungi infect plants through wounds or directly via stomata or penetration of the surface layers. In leaves this barrier is further enhanced by the presence of a sometimes thick and waxy cuticle. Before the development of systemics, all fungicide compounds were non-systemic protectants, effecting disease control only through their activity on the plant surface. Characteristically, after application to foliage these compounds control disease either by killing superficial mycelium, as for example in the powdery mildews that penetrate only the topmost cellular layer, or more commonly by preventing the germination of fungal spores already present on the leaf or impacting on the leaf after application. Non-systemics cannot penetrate the leaf and hence cannot control pathogens already established within the plant tissue. Therefore, foliage must be treated before the pathogen has colonized the plant. Subsequent development of the plant exposes new tissues to fungal attack and may rupture protective fungicide deposits. Hence, such products must be applied frequently during the growing season to maintain acceptable disease control levels. Although the lack of mobility of early fungicides limited their flexibility of use, their inability to penetrate plant tissue allowed them to exploit the control spectrum inherent in their non-specific biochemical mode of action (MOA). This remains a valuable feature in their current uses against a broad range of pathogens and in strategies to control resistance to systemic fungicides.

The introduction of systemic compounds caused a revolution in farmer practice and in fungicide discovery and development. New opportunities for fungicides were immediately identified, as in intensive cereal production in Western Europe. Fungal diseases of wheat and barley had been a disturbing feature of cereal production for at least 2000 years but the use of resistant varieties, stimulated in part by the failure of early products to control pathogens such as mildew and rust, enabled infection to remain at an acceptable level. The associated yield losses were estimated to be insignificant until systemic fungicides were discovered and tested, beginning with the morpholines ethirimol and tridemorph.

Field trials demonstrated that the yield benefits that could be achieved using the new fungicides were on average about 10%. Yields increased further as the limits of varietal potential were explored using combinations of higher fertilizer inputs and fungicides. European Community legislation in the 1970s–1990s encouraged high-output production systems, and inputs such as the use of high levels of fertilizers and pest control chemicals increased to maximize yields. The rate of discovery of new and more effective fungicides also increased, and in 20 years the range of foliar and ear diseases for which some control could be claimed had expanded from a few seed-borne pathogens and mildews to include cereal rusts caused by various *Puccinia* species, wheat septoria nodorum blotch, septoria tritici blotch, Fusarium head blight and eyespot, the barley diseases net blotch and scald, and the maize Cochliobolus leaf blights.

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The new products afforded better levels and duration of control and allowed the grower more flexibility in application. However, even they failed to provide complete disease control, and the search for more effective materials and technology continues.

The appearance of systemic fungicides and the increasing variety of products available to the grower corresponded with the requirement of the fungicides industry to adopt new and higher standards of performance. The most important was, and remains, safety. This arose from the general acceptance by the industry of the need to avoid a repeat of the damaging impact of early organochlorine insecticides like DDT, highlighted by the publication of Rachel Carson's book *Silent Spring* (Carson, 1962). Hence, much greater efforts were made to ensure that the products were safe to the manufacturer, the user, the consumer of treated crops and all aspects of the environment. The industry and government registration authorities became responsible for the development of only those materials proven to be safe and environmentally acceptable. In addition, to compete successfully, product attributes other than biological activity assumed major roles (Table 1.4).

Despite these efforts, distrust in the safety of agrochemicals generally has remained prevalent in a significant section of the population, especially in richer and more urban communities. This has in turn fuelled the growth of the 'Organic' or 'Biological' agroecological movements. This style of farming seeks to avoid the use of synthetic agrochemicals and instead relies on the use of a small group of inorganic fungicides (such as copper and sulfur), natural products and BCAs (mainly bacteria) for disease control.

Table 1.4. General targets for new fungicidal products.

Attribute	Type of product improvement
Safety	Safe to users Environmentally acceptable Safe to consumers of the treated product
Performance	Broader disease-control spectrum Extended control period Increased reliability Anti-resistance activity Improved crop safety
Use	Compatibility with other products Easy-to-use formulations Safe application
Cost	Lower cost per treatment through the use of: cheaper fungicides lower use rates fewer treatments per season lower application costs

The number of products and mixtures grew to meet the new market standards of disease control. In the triazole family alone there are on average about ten products – different formulations of solo active ingredients (AIs) and mixtures – per compound. Many fungicides appear to increase yield beyond that attributable to the reduction of disease. Late-season treatment with benomyl, an early systemic fungicide, was shown to delay senescence and increase yield by up to 10% through a combination of fungicidal action and plant growth regulator effects. Similar activity is reported for QoI and SDHI fungicides and although the cause is unclear, it is thought to be associated with the control of phylloplane organisms and a direct effect on the maintenance of photosynthetic ability.

There is little doubt that the intensive monoculture-based agricultural systems that are needed to provide the growing population with food also encourage fungal disease epidemics, and the removal of fungicides from agriculture does not appear to be a realistic option. The emergence of fungicide resistance and the need for more cost-effective products encourage the search for better remedies, whether they be synthetic products or materials derived from natural sources or through the introduction of genetic modification of target crops.

The Growth of the Agrochemicals Industry

Pesticides, synonymous with agrochemicals or crop protection agents, comprise mainly herbicides, insecticides, fungicides and plant growth regulators. Further definition can be confusing. A pesticide is strictly an agent that kills a pest and can be either synthetic or natural. However, the definition omits plant growth regulators, which are designed to enhance the growth and

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development of crops directly. In addition, the term pesticide is often applied only to insecticides. Pesticides are better classified as agents that maintain the yield potential of crops under adverse growing conditions, caused by the presence of weeds, pathogens or insects. Under this definition, pesticides are products that combat biotic stresses.

Agrochemical companies developed as a diversification of those chemical industries specializing in the manufacture of organic dyestuffs. Originally including the fertilizer industry, the agrochemicals business is now distinct and comprises a large, high-value, high-technology industry that survives upon innovation and the discovery and development of synthetic and natural pesticidal products. Despite the success of the pesticides business, the industry is shrinking. The conflicting forces of price competition, affecting margins and profitability, and the increasing costs of discovery and development of potential products and the maintenance of established pesticides have resulted in a phase of consolidation. The situation was made more acute through the increased political and social recognition of the environmental issues associated with pesticide use and the subsequent demand for more extensive product examination. This led to spiralling increases in the costs of safety testing, the prolongation of development time and a subsequent reduction in effective patent life. A shorter product lifespan and the need to generate a return on a rapidly increasing research and development investment have stimulated the search for economies of scale such that the agrochemicals industry is now dominated by a few large international companies. Just 25 years ago there were ten major international fungicide companies. Now there are only four major players active in all phases of fungicide discovery, development, manufacture and sales. These are Syngenta (now merged with Chemchina), Bayer CropScience (incorporating Monsanto), BASF and Corteva (formerly Dupont and Dow). A larger number of companies manufacture and sell fungicides either that are no longer protected by patents or in collaboration with the four majors. These companies are called generic manufacturers.

Fungicides form a vital part of the research effort and product ranges of all major agrochemical companies, driven by their well-established use in a wide variety of globally important crops. Their markets, discovery and use, and the legislation that governs their development, are presented in the following chapters.

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2

Plant Pathology and Plant Pathogens

Key Points

- What is a disease?
- A diverse range of organisms causes diseases of plants.
- Two types of pathogens dominate, the *Oomycota* and the *Fungi*; these are the targets of the great majority of fungicides.
- Fungi are more related to animals and oomycetes are more related to plants than either is to the other. This is reflected in the different fungicides that control each group.
- Pathogens are divided into biotrophs and necrotrophs and an intermediate class, hemibiotrophs.
- Effectors are pathogen molecules that interact with plants and contribute to the disease phenotype.
- Biotrophs and necrotrophs have different types of effectors and induce different plant responses.

Introduction

Fungicides work by inhibiting the infection processes used by pathogens to cause disease or by enhancing the defensive capabilities of the plant. A very large range of organisms causes disease on plants but first we must define 'disease'. We can operationally define disease as the ability of a pathogen to reduce the yield and/or the quality of a crop while growing and reproducing on the host plant. Pathogens are defined as organisms that cause disease. They are distinguished from saprobes and symbionts. Saprobes (previously saprophytes) are species that live off dead material and are ecologically limited to that role. Symbionts are organisms that grow on or in the plant and that are beneficial to its growth. This group includes the mycorrhizae, fungi that form close associations with roots and benefit the plant by making phosphate more available, and the rhizobia, bacteria which supply nitrogen to leguminous plants. We are also becoming much more aware of the vast complexity and abundance of microorganisms that associate with plants without causing substantial impacts. The microbiome of the plant, as this group is known, has only recently been studied in detail (Berendsen *et al*., 2012). It is likely that the microbiome is important for plant health although the details are not yet clear. From the perspective of the farmer, a key characteristic of a good fungicide is that it controls the microorganisms that cause disease, the pathogens, while having no negative impacts on symbionts or the more general microbiome.

Pathogens can be subdivided into obligates and non-obligates which are also called facultative pathogens. Obligate pathogens can only grow

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and reproduce on living hosts, whereas facultative organisms are also capable of growth and reproduction on dead material including artificial media in laboratories. This has important implications as non-obligates are much easier to study under controlled conditions.

Pathogenic species are found in many groups of organisms and include viruses, phytoplasmas, viroids, nematodes, parasitic plants, algae, trypanosomatids, bacteria, *Fungi*, *Oomycota* and *Plasmodiophora* (Strange, 2003). These groups encompass much of the biological diversity found in life. Unsurprisingly, no one strategy can control diseases caused by each of these groups. An understanding of the diverse ecological and biochemical properties of these groups is needed to appreciate the potential for chemical disease control.

The first seven groups – viruses, phytoplasmas, viroids, nematodes, parasitic plants, algae and trypanosomatids – are not considered further in this book as chemical agents for their control are currently not significant. Some groups are transmitted by insects and so are controlled with insecticides. Bacteria cause major diseases in some situations, and they can be controlled both by genetics and by chemicals. The chemicals are typically known as antibiotics, reflecting their origin in animal and human therapeutics, or occasionally as bactericides (Sigee, 2005). Being prokaryotic, bacterial antibiotics rarely have any activity against other types of pathogens. Antibiotics and insecticides are not considered further in this book.

The remaining groups are all microbial eukaryotes – that is, organisms too small to be seen with the naked eye, which share basic biochemical features with animals and plants, and which differ in fundamental ways from the various prokaryotic bacterial groups. The defining feature of eukaryotes is that they contain nuclei and most carry other organelles such as mitochondria.

Until recently, the phylogenetic relationship between the different groups of eukaryotic microbes has been problematic and poorly understood. Difficulties of cultivation and the limited availability of morphologically meaningful features have hindered progress. Knowledge on the evolutionary history of eukaryotic microbes has undergone a revolution in recent years as a direct result of advances in molecular biology and DNA sequencing as applied to phylogenetics and taxonomy. We now have a good understanding of the deep evolutionary differences between these organisms and can now rationalize differences in activity of fungicides against these species (Adl *et al.*, 2005; Keeling *et al.*, 2005). Although many of the species are not fungi, all compounds that control these species are normally referred to as fungicides and will be covered in this book.

Table 2.1 lists the higher-level taxa in which are found the major groups of pathogens and their hosts. Although the details of the highest level of taxonomy are still subject to revision, fungi and animals are relatively closely related and these are very distantly related to the *Oomycota*, *Plasmodiophora* and their plant hosts. This modern view of taxonomy emphasizes that pathogenicity has arisen in multiple and diverse taxonomic groups. It also emphasizes the difficulty of finding compounds that have good spectrum (i.e. that control a broad range of pathogens) but do not damage either the host plant (known as phytotoxicity) or non-target organisms, such as insects and the human population.

Table 2.1. Taxonomic placement of the major groups of microbial eukaryotic pathogens and key non-target groups, animals and plants. Non-target groups are in bold. (Modified from Oliver and Hewitt, 2014.)

Clade	Phylum	Examples
Viridiplantae	*Streptophyta*	**All plants**
Opisthokonta	*Fungi*	
	• *Basidiomycota*	*Puccinia*
	• *Ascomycota*	*Blumeria*, *Magnaporthe*, *Ascochyta*
	• *Glomeromycetes*	**Mycorrhizae**
	Metazoa	**All animals including nematodes**
Rhizaria	*Endomyxa*	*Plasmodiophora*
Stramenopiles	*Oomycota*	*Phytophthora*, *Pythium*, *Peronospora*

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Characteristics of Plant Pathogens

Fungi

The *Fungi* are by far the most important group of plant pathogens especially in terms of the number of species and the variation in their pathogenic lifestyles, but also in incidence and damage. It is no coincidence that compounds that control plant diseases are called fungicides.

All plant pathogens are heterotrophic. This means they adsorb small-molecular-weight nutrients from the external medium. They typically secrete enzymes into the external medium that break down complex polymeric plant materials – proteins, carbohydrates and lipids – into small molecules that they can directly adsorb. These include amino acids, sugars and fatty acids.

Most pathogens are filamentous and grow by extending a hyphal tip. The hypha is divided into cells by septa. Some fungi grow as yeasts, single-celled organisms growing by cell division. All fungal pathogens have rigid cell walls with chitin as the major strengthening compound. This distinguishes them from oomycetes that have cellulose-based cell walls, like plants. The cell membranes of fungi contain the sterol ergosterol, in contrast to animals which have cholesterol and plants and oomycetes which have more diverse 'phytosterols' that are derived from their plant hosts. Ergosterol and cellulose biosynthesis are the targets of major groups of fungicides. The presence of these components in different groups of pathogens explains the limited spectrum of these fungicides. Unlike oomycetes, fungi lack flagella and are incapable of directional movement except via hyphal growth.

Fungi reproduce by producing spores. These can be either or both asexual and sexual structures. Traditionally the taxonomy of fungi has depended on the discrimination of morphological features of spores. As many species produce both sexual and asexual spores, a single species often had two names: a teleomorph based on the structure of the sexual spores (often called the perfect state) and an anamorphic name based on the asexual spores (called the imperfect state). Fungi that were not known to produce sexual spores used to be called the *Fungi Imperfecti* or *Deuteromycota*; this hid their real evolutionary relationships to 'perfect' fungi. Furthermore, as the taxonomy was based on sparse morphological data that had a degree of subjectivity, different authors would suggest different names. As a result, few fungal pathogens had a single agreed name, resulting in confusion not only among pathologists and growers but also quarantine authorities. Recently, the official bodies have agreed to a system whereby each species has only one name. Where more than one exists, the oldest published name should be used. This ends the automatic priority of names of teleomorphs over anamorphs (Hawksworth *et al*., 2011). This change should be greeted with relief by the fungicide community which is traditionally reluctant to adopt new names. None the less, species are still known by several names and Table 2.2 lists some of the most important as well as the abbreviations used in the fungicide industry.

These changes have been brought about very largely because of the ease of acquiring and interpreting molecular data on DNA and RNA sequences compared with morphological or chemical data. The same data sets are being used to create phylogenetic trees. This eliminated the *Deuteromycota* and substantially revised the deeper phylogenetics of the fungi (Spatafora *et al*., 2017; Naranjo-Ortiz and Gabaldón, 2019).

The fungi are divided into six major groups of which the *Ascomycota* and *Basidiomycota* are the most important, although there are important pathogens in the *Chytridiomycota*. Chytrids mainly infect animals, but a few infect plant species; in particular, maize- and lucerne-attacking species have been described. The *Zygomycota* include the symbiotic mycorrhizal fungi (also called *Glomeromycetes*) and hence are an important beneficial group that could be deleteriously affected by fungicides.

The *Ascomycota* is the biggest phylum and contains most of the important pathogenic species. It includes the mainly filamentous subphyla – the *Pezizomycotina* – and two yeast groups. There are few pathogens among the yeasts. Instead, yeasts can be regarded as beneficials especially in the fermentation industries; care must be taken that fungicides used to control diseases do not interfere with wine or beer fermentation by wild or inoculated yeasts.

The higher-level classification of the filamentous *Ascomycota* remains highly fluid but some stable groups, which include most of the plant

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Table 2.2. Abbreviations and names of some major pathogens and diseases. (Modified from Oliver and Hewitt, 2014.)

EPPO abbreviation	Disease	Host	Preferred name of pathogen	Synonym(s)
ERYSGH	Powdery mildew	Barley	*Blumeria graminis* f. sp. *hordei*	*Erysiphe graminis* f. sp. *hordei*
ERYSGT	Powdery mildew	Wheat	*Blumeria graminis* f. sp. *tritici*	*Erysiphe graminis* f. sp. *tritici*
BOTCRI	Botrytis; grey mould	Many, especially grapevines	*Botrytis cinerea*	*Botryotinia fuckeliana*
GIBBZE	Head blight	Wheat	*Fusarium graminearum*	*Gibberella zeae*
GIBBFU	Bakanae disease	Rice	*Gibberella fujikuroi*	
PYRIOR	Blast	Rice and wheat	*Magnaporthe oryzae*	*Magnaporthe grisea*; *Pyricularia grisea*
MYCOFI	Black sigatoka	Banana	*Mycosphaerella fijiensis*	
LEPTNO	Septoria nodorum blotch	Wheat	*Parastagonospora nodorum*	*Phaeosphaeria nodorum*; *Septoria nodorum*; *Leptosphaeria nodorum*
PHAKPA	Rust	Soybean	*Phakopsora pachyrhizi*	Asian rust
PHYTIN	Late blight	Potato	*Phytophthora infestans*	
PLASVI	Downy mildew	Vine	*Plasmopara viticola*	
PODOFU	Powdery mildew	Apple	*Podosphaera leucotricha*	
PUCCRT	Brown rust	Wheat	*Puccinia recondita*	
PUCCST	Yellow rust	Wheat	*Puccinia striiformis*	Stripe rust
PYRNTE	Net blotch	Barley	*Pyrenophora teres*	*Drechslera teres*
PYRNTR	Tan spot	Wheat	*Pyrenophora tritici-repentis*	*Drechslera tritici-repentis*; yellow spot
UNCINE	Powdery mildew	Vine	*Uncinula necator*	
VENTIN	Scab	Apple	*Venturia inaequalis*	
SEPTTR	Septoria tritici blotch	Wheat	*Zymoseptoria tritici*	*Septoria tritici*; *Mycosphaerella graminicola*
PSDCHE	Eyespot	Wheat	*Tapesia yallundae*	*Pseudocercosporella herpotrichoides*; *Oculimacula yallundae*

EPPO, European and Mediterranean Plant Protection Organization.

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pathogens, have emerged. The new phylogeny groups together some organisms in a biologically relevant way but it is also clear that fungi from different groups share apparently common features. The order *Dothideomycetes* includes the class *Pleosporales* that includes most of the species known to produce necrotrophic effectors: *Cochliobolus*, *Alternaria*, *Pyrenophora* and *Parastagonospora* (Oliver and Solomon, 2010). In contrast, it is surprising that the archetypal host-specific biotrophic pathogens, the powdery mildews (*Blumeria* and *Erysiphe*), and the archetypal non-host-specific necrotrophs, *Botrytis* and *Sclerotinia*, are combined in the class *Leotiomycetes*. Species with a hemibiotrophic lifestyle are found in the other classes of the *Dothideomycetes* (e.g. the major wheat pathogen *Zymoseptoria tritici* and the major apple pathogen *Venturia inaequalis*) as well as the *Sordariomycetes* (e.g. bean anthracnose, *Colletotrichum lindemuthianum*; rice blast, *Magnaporthe oryzae*; and the Fusarium wilt pathogens).

The *Basidiomycota* include just two major groups of pathogens: the *Ustilaginomycotina* and the *Pucciniomycotina*. Both groups figure heavily in histories of plant pathology and continue to cause major losses. The *Ustilaginomycotina* include the bunts and smuts which include mainly seed-borne and flower pathogens of cereals. *Ustilago maydis* is an important model organism. The control of seed-borne bunts and smuts by fungicides is one of the great success stories of the chemical industry. Resistance problems are very rare. The main issue with these diseases is that because the chemicals work so well genetic resistance can easily be neglected.

The *Pucciniomycotina* include the infamous rust diseases that have for so long been the scourge of growers that they were mentioned in the Bible. All rusts are typical biotrophic pathogens showing a high degree of host specificity and the inability to be cultured on media.

Oomycota

The other major group of pathogens is the *Oomycota*. Oomycete diseases typically require wetter conditions than fungi; hence their old name, the water fungi. This group includes several highly destructive and historically significant pathogens. The most famous example is the potato late blight pathogen *Phytophthora infestans* that triggered the great 1845 Irish potato famine; it still causes major losses today and is a major target for fungicide development. The other two groups are *Pythiales* and *Peronosporales*. *Pythium* species are the cause of seedling damping-off diseases, whereas the *Peronospora* cause the downy mildews. The diseases caused by *Oomycota* include many that can be described as biotrophic, such as the downy mildews, as well as hemibiotrophic interactions, such as those caused by the *Phytophthora* group.

At first glance, these three groups share many of the features of fungi. They are eukaryotic, heterotrophic, acquire nutrients only by adsorption and grow by filamentous expansion. They cause diseases with mildew, blight or rot symptoms just like fungi. However, there are also obvious differences. They have motile spores that use flagella. They lack chitin and ergosterol and instead have cellulose-reinforced cell walls with phytosterols in their cell membranes. And importantly, most of the fungicides that work against oomycetes do not control fungi and vice versa. These differences were resolved once molecular phylogenetic data were applied to eukaryotic taxa (Forster *et al*., 1990). These data clearly showed that oomycetes were completely unrelated to fungi. Indeed, fungi share a common ancestor with animals and if anything, oomycetes share more common features with plants.

Plasmodiophora

A common root disease of brassica crops called clubroot is caused by *Plasmodiophora brassicae*. This organism has been placed into a distant taxon, the *Rhizaria*. It was previously known as a slime mould and placed with the ‘protists’. Fungicides are generally ineffective, not least because it is a soil pathogen.

Phytopathogenic lifestyles; biotrophs, necrotrophs and hemibiotrophs

Plant pathologists have traditionally divided pathogens into two broad classes: biotrophs and necrotrophs. The suffix ‘-trophy’ relates to the mode of nutrition of the pathogen during infection. The definition of biotrophy is that the

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pathogen requires living host cells to acquire nutrients; in contrast, necrotrophs can complete their life cycle on dead or dying material. The two classes are associated with several other characteristics. Biotrophs tend to be obligate – that is, they cannot be grown in culture. It is still accepted that all obligates are biotrophs, but the reverse has exceptions. Biotrophs tend to be host-specific and to be well controlled by major resistance genes unless the resistance breaks down. This tendency to overcome resistance genes – the boom and bust cycle – was conceptualized by Flor into the gene-for-gene hypothesis (Flor, 1956; Keen, 1990). The feeding of obligate biotrophic fungi is always associated with a specific feeding structure, a haustorium. Resistance to these pathogens is linked to the synthesis of salicylic acid which induces defence responses in both local and distant plant tissues. In contrast, necrotrophs can always be grown in culture, often have a broad host range and genetic resistance tends to be partial. Necrotrophs often produce copious cell-wall-degrading enzymes in culture and toxic compounds that promote disease. Resistance is more likely to be associated with accumulation of jasmonic acid and to resemble defence against insects and wounding (Oliver and Ipcho, 2004).

Many pathogens do not fit neatly into either class, and some are formally classified as hemibiotrophs – pathogens that exhibit both biotrophic and necrotrophic characteristics. These phases can be differentiated in time (first biotroph and then necrotroph) or space (initial penetration and establishment is biotrophic but once a deeper tissue is reached, the fungus becomes necrotrophic).

Fungicide sensitivity is not correlated with whether a fungus/oomycete is described as a biotroph, necrotroph or hemibiotroph. Instead, taxonomic placement has turned out to be a much better predictor.

Avirulence genes, PAMPs, MAMPs and effectors

The application of molecular genetics tools to plant pathology has gathered pace since the 1980s and has now generated a body of knowledge that broadly explains the different types of pathogens – biotroph, necrotroph and hemibiotroph – and how they interact with their plant hosts (Koeck *et al.*, 2011; Vleeshouwers and Oliver, 2014; Couto and Zipfel, 2016; Toruño *et al.*, 2016). The new paradigm revolves around the concept of pathogen-associated molecular patterns (PAMPs) (also known as microbe-associated molecular patterns (MAMPs) and pathogen 'effectors') and the nature of the plant's response. Effectors are defined as molecules produced by the pathogen that interact in a specific way with the plant so as to produce a reaction that has a bearing on the outcome of the disease; effectors affect the plant and effect disease. PAMPs are molecules produced uniformly by multiple classes of microbe and are detected by the plant using specific receptors. Recognition induces the plant to produce an immune response. Specific effectors are found only in particular species or strains of a pathogen and act to suppress the PAMP-induced defence response. If plants recognize the specific effector this can lead to a successful defence response. Such proteins are now called biotrophic effectors; they were previously called avirulence (avr) genes. This name derived from the finding that resistant plants evolved the ability to recognize the effector and induce an effective defence response. Loss of the recognition by loss or alteration of the effector allowed the pathogen to cause disease once again. Hence, in formal genetic terms, the effector operated as a pathogen molecule that prevented disease; an avirulence gene. Recognition of the avr gene product by the plant was done by resistance genes. Hence resistance was dominant. Necrotrophic effectors (NEs), on the other hand, are recognized by specific receptors in the plant but unlike biotrophs, the defence response to NEs allows the pathogen to enter deeper into the plant and to acquire nutrients from dead and dying tissues.

Genomics and genetic variability in plant pathogens

The development of molecular biological techniques has had a profound impact on the study of plant pathogens, the discovery of fungicides and the understanding of fungicide resistance (Cools and Hammond-Kosack, 2013). Early studies had shown that both fungi and oomycetes were capable of rapid adaptation to overcome new genes for disease resistance and new fungicides, but the mechanisms were unclear. The

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critical factor has been the spectacular increase in the power and efficiency of techniques to detect genetic variations between isolates of a pathogen. A procession of techniques has been used to study variation – RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), SSR (simple sequence repeat), SNP (single-nucleotide polymorphism), HRM (high-resolution melt analysis) – but for many purposes these have been superseded by the modern methods to sequence entire genomes. It is now straightforward and relatively inexpensive to sequence several hundred isolates of pathogens and compare them with a reference genome. Differences in the phenotype of reference and test isolates can then be linked to differences in the genome.

Both fungi and most oomycetes have relatively small genome sizes and numbers of genes compared with animals and plants. However, they are capable of very fast adaptation because their genomes have great plasticity. As a result, pathogen populations can evolve to overcome new resistance genes or new fungicides in a matter of a few years at most and a few days at least. The reasons for this genomic plasticity lie in the life-cycle details of pathogens and genomes that can generate substantial genomic alterations.

All the pathogens we study produce very large numbers of spores (or other propagules) during the course of a successful infection. Although a few pathogens complete their life cycles once a year, the great majority can reproduce on their hosts multiple times in a growing season. Furthermore, many are capable of both asexual reproduction and sexual reproduction. Thus, any strain that mutates to overcome a resistance gene or fungicide can increase in population size very rapidly. The genomes of plant pathogens contain large amounts of apparently non-coding DNA, much of which appears to be active or inactivated transposable elements. These regions are hotspots of variation occurring during both mitotic and meiotic nuclear division. Large chunks can be duplicated or lost at each nuclear division. The number of sizes of chromosome can vary markedly even between the progeny of a single cross. The variation between the gene-rich stable parts of the gene-poor unstable regions gave rise to the concept of the 'two-speed' genome, sometimes called 'genomic tillage'. The high-speed, gene-poor regions nevertheless often contain key effector genes. Placement in the high-speed regions of genomes facilitates many mutagenic processes based on errors in replication. Thus, these regions can be regarded (somewhat inaccurately) as 'gene workshops'. Duplications of small regions of genome are also often observed (merodiploidy) and this has been correlated with some cases of fungicide resistance (Oliver, 2012; Dong *et al.*, 2015).

The presence of transposable elements opens more possibilities for genomic variation. The movement of sections of DNA around the genome can inactivate genes, when the transposon lands within it, or activate them, when it lands in the promoter. The former process is important in inactivating avirulence genes while the latter can lead to overexpression of fungicide target genes. Genes can also be acquired from outside the organism. Horizontal (or lateral) gene transfer can shift genes into species, giving them enhanced virulence or new hosts. The movement of transposons and the acquisition of horizontal genes is countered in some fungi (mainly ascomycetes) by a process called RIP (repeat-induced point mutations). If duplicated genes are present when it undergoes meiosis, the machinery seeks out both copies and substantially mutates them. Normally both genes are inactivated but occasionally one, highly mutated copy escapes. Also, the process is somewhat leaky and neighbouring genes can be altered. Hence RIP can unleash a hotspot of mutation in a region already containing introduced DNA and transposable elements.

For all these reasons – rapid life cycles, asexual and sexual reproduction, horizontal gene transfer, transposons and RIP – many pathogens are subject to a plethora of hypermutating processes. These processes allow the production of new strains which can be acted upon by the selection pressure exerted by new resistance genes and fungicides (Oliver, 2012; Dong *et al.*, 2015).

The Impact of the New Paradigms on Fungicide Research

The resolution of the previous confusion in pathogen names, pathogen types and pathogenicity mechanisms has explained many previous inconsistencies in fungicide performance. The clarification of the gulf between fungi and

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oomycetes has helped explain fungicide spectrum. Spectrum is the term used to describe the range of pathogens controlled by a particular fungicide. The resolution of the confusion between obligates and non-obligates versus biotrophs has impacted on the way fungicides are discovered and developed (for details see Table 4.5 in Chapter 4, this volume). In one case, the fungicide Bion (acibenzolar-*S*-methyl; ASM) that operates by potentiating the salicylic acid defence response, its efficacy mainly against biotrophic pathogens is now understandable.

Nomenclature in the Literature and Practice

Plant pathology is beset with a confusing set of nomenclature rules. Each pathogen that causes an important disease can have a variety of names. As we have seen, the fungal nomenclature rules have changed substantially in the last few years, but several different names persist for most if not all fungi. In addition, the disease can have several names; for example, tan spot is known as yellow spot in some countries, yellow rust is also known as stripe rust. The fungicide industry has adopted a six-letter abbreviation for some of the more important diseases and pathogens (see Appendix – EPPO Codes, this volume). The abbreviation is based on a binomial that existed at one point in history; what we now call *Blumeria graminis* f. sp. *tritici*, but used to call *Erysiphe graminis* f. sp. *tritici*, has the abbreviation ERYSGT. In this book, we shall use the six-letter code when it exists and the preferred pathogen and disease names when it does not.

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3

The Fungicide Industry

Key Points

- Fungicides are discovered and marketed mainly by large, international, private businesses.
- The discovery and development of a new fungicide is expensive and risky.
- New fungicides are covered by patents but once these expire, other companies can produce and sell generic versions.
- Sales of major fungicides need to amount to around $1000 million ($1 billion) to recoup costs.
- Fungicides are sold to nearly all countries; sales in middle-income countries are rising sharply.
- Cereals, fruit and vegetable crops, grapevines, soybean, rice and pome fruits make up 85% of fungicide use.

Introduction

The discovery, development, production and marketing of fungicides has been undertaken almost exclusively within the private sector, by large, independent and multinational companies. In contrast, plant breeding and extension activities, which support genetic and cultural disease control methods, were until recently mainly in the domain of state agencies and universities. Fungicide discovery and development has received only very limited public-sector support, mainly through co-investment in upstream research. Thus like all private-sector enterprises, a company producing fungicides needs to provide a satisfactory rate of return for its investors and to generate resources essential to company growth and development.

The agrochemicals business is capital-intensive, and the companies continually review their commercial objectives. They focus their activities on targets and markets that are large enough to support the costs of the development of new products. These may include existing markets that are dominated by products that are vulnerable to deregistration for environmental safety reasons or are suffering from a decline in efficacy due the evolution of resistance. They may also include new markets where pathogens have crossed national or host species boundaries and generated a new disease threat.

Fungicide targets and their priorities in the discovery process are defined not by their biology, but by their economics. The exercise of target definition is straightforward and common to all companies, the only differences between companies being the level of return or risk deemed to be acceptable in the pursuit of a particular market goal. For example, the key disease driving much fungicide development in the past

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30 years has been septoria tritici blotch of wheat (caused by SEPTTR) (Fones and Gurr, 2015). As much as $1 billion per annum is spent on fungicides to control this disease in Europe alone. Furthermore, this fungus seems to be particularly adept at evolving resistance, so many individual AIs have been successively released to counter this threat. Newly emerged diseases are also important drivers of fungicide development as it is often quicker to deploy fungicides than to breed disease resistance cultivars. A good example of this is Asian soybean rust since it spread to South America (caused by PHAKPA) (Yorinori *et al.*, 2005; Furlan *et al.*, 2018).

What level of return is required by industry for the control of a particular disease problem to become an acceptable commercial target? To answer that question, it is necessary to understand the costs involved in the discovery and development process, and to appreciate the effects of financial thresholds that companies impose upon the sale of products. Candidate fungicides enter the process of biological evaluation and commercialization from various sources and range in cost from several hundred to many thousands of dollars each. Passage through the screening and development system eliminates most candidates, with approximately one commercial product emerging for every 160,000 compounds screened (Phillips McDougall, 2016), a much lower success rate than before (Table 3.1). This industry-wide measure of success worsens annually as new materials that meet increasing demands of performance, competition and legislative restrictions become more difficult to discover.

The current industry average cost for the development of a new fungicide is approximately $286 million to $300 million, committed over a period of over 11 years, up from 8 years in 1995, prior to product launch (Table 3.1). Two-thirds of the total cost is attributed to biological efficacy trials and exhaustive toxicological and environmental safety tests. There was a sharp rise in the costs associated with this phase between 2000 and 2008 primarily due to the increasingly stringent environmental chemistry costs. The cost of developing a fungicide has increased from about $80 million in 1976, highlighting the contribution of compliance with increasingly stringent regulatory requirements.

Companies almost always take out a ‘Letters Patent’ to protect their new invention. The Patent lasts up to 20 years (see Box 3.1) dating from near the beginning of the 10+-year development period. A new product may not show an operating profit for at least 2 years after commercialization. Thereafter, there may be only a few years of patent protection in which to recoup the research and development investment costs on all compounds tested, including those that failed at some point in the development process. Companies can expect a few years of maximum profit, before having to contend with direct competition after patent expiry. Thereafter generic manufacturers may seek to enter the market, leading to lower prices.

The prominence of fungicide resistance has changed the way new fungicides are marketed. It is now recognized that overuse of a fungicide is one of the main driving forces behind the evolution of resistance. Fungicide companies must therefore resist the temptation to sell as much as possible of their new AI as soon as possible after product launch and during the period of patent protection. Instead, new fungicides are marketed with caution and limits are placed on the number of times they can be used within a season on each crop. Very often they are sold in mixtures with other actives. The key parameter is now longevity – how long can a fungicide remain commercially viable. A good example of this is the world’s current biggest selling fungicide, the QoI azoxystrobin. Azoxystrobin was announced in 1992 by ICI (now owned by Chemchina/Syngenta) and marketed from 1996. Each of the major companies had a QoI on the market by 2000.

Table 3.1. The increase in the number of compounds screened per year and the time taken to achieve successful registration in Europe. (From Bryson and Brix, 2019, with permission.)

	1995	2000	2008	2014
Compounds screened	52,500	139,429	140,000	159,574
Lead time (years)	8.3	9.1	9.8	11.3

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Box 3.1. Patents and intellectual property.

The patenting system has a bad press among the public, but without it, it is hard to see how we could have access to any of the technological advances, from pharmaceuticals to transport to communications, that make up our modern world. The patenting system is central to the operation of the fungicide companies and an understanding of the basic principles helps explain the nature of the industry.

The purpose of the patenting system is to encourage innovation in all manner of products and services. It does this in three main ways; first, it grants an inventor time to exploit their invention during which only the inventor can make and sell the product. Second, it forces the inventor to disclose full details of the invention so that competitors can benefit from the underlying knowledge. Patent means ‘open’; the alternative would be secrecy. Third, it forces an inventor to use a patented invention; failure to do so can result in the granting of licences (permissions) to other parties to develop the idea.

The patenting system operates via government agencies called Patent Offices. The European Union has a single office while most other countries have their own. Many countries are signatories to patent treaties that bind themselves to abide by the common principle of respecting the patent system and the free trade of products.

The process of patenting starts when an inventor submits a ‘Provisional Application’ to the local patent office. The ‘inventor/s’ are named individuals within the fungicide company, a university or a private individual and they normally gift or sell (‘assign’ in the jargon) the invention to their employer or another fungicide company at some stage in the process. The Provisional is typically a short document describing the invention and is cheap to file and process. The Provisional establishes a ‘priority date’. Other inventors working in the same area but with later priority dates will be in a much weaker position. Just a few days separated the filing of patents describing the first two QoI fungicides. On the other hand, the priority date starts the process that will, in due course, result in the ending of the protection. Typically, provisional applications are filed prior to the full development of the invention. The document is not made public, but the inventor can disclose it to organizations to try and secure the financial backing to develop the invention; these might be fungicide companies or venture capitalists, research agencies or charities. If such an organization were interested, it might buy the invention and fund the research, granting the inventor a royalty or some other recompense.

The Patent Office will examine the patent and determine whether the invention satisfies the criteria of patentability; these are novelty, non-obviousness and utility. Novelty is determined by reference to published material, whether other patents, academic papers or the general literature. These are collectively called the ‘prior art’ and lie in the ‘public domain’. The non-obviousness criterion is designed to disallow trivial improvements. Utility is defined as conforming to natural laws (i.e. perpetual motion machines are not patentable) and being capable of commercial exploitation.

Provisional patents last only 1 or 2 years and during that period the relevant Patent Office does not examine the document. If the inventor (or the new owner) wishes to pursue the patent, increasingly large fees need to be paid to the Patent Office and expensive patent attorneys are typically needed to draft the full descriptions of the invention in the patent application. Furthermore, the inventor or owner must file the patent in all countries in which they would like protection. New treaties are making this international filing more straightforward.

The most important element of the description is the section called the ‘Claims’. Key fungicide patents are typically descriptions of chemicals that can be marketed safely and economically to control a range of diseases. It is likely that, at first, only a single compound is known to the inventor and described in detail. However, nearly all fungicides fall into classes of similar compounds that share a common structural feature and a common MOA. It might well be futile to patent just a single compound. All a competitor would have to do, following disclosure of the patent, is alter the compound in a variety of trivial ways, find a variant with activity and patent that. The competitor would have saved the huge costs of chemical discovery and the inventor would find their market diminished. Hence the inventor will tend to inflate their discovery and claim the use of all chemically related compounds and all compounds that operate via the new MOA. The claims could relate to many compounds that may not even have been synthesized. In contrast, the Patent Office, encouraged by competitors, will insist that only tried and tested compounds are included. This tension is central to the day-to-day life of fungicide companies as they seek to outflank each other’s patents.

Continued

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Box 3.1. Continued.

Eventually the Patent Office may grant the Letters Patent. The owner of the invention now has a specified period, typically 16 or 20 years from the time of the Provisional, for exploitation. In practice however, development of the patent may have taken 5–10 years so the effective period may be only 10 years or less. During this period, the inventor not only needs to recoup the cost of manufacture and distribution, but also of research and development.

After this period, the compound(s) go 'off patent' and anyone can legally make and sell the product. They will have the benefit of full details of the manufacturing process upon which to base their version of the product. The price will inevitably drop.

Some companies avoid the process of discovery altogether and choose to specialize in the manufacture of so-called 'generic' products. Furthermore, some countries do not operate a patent system and thus feel free to manufacture any product at any time. They are prevented from selling their products in countries that operate within the patent system by fear of sanctions from the World Trade Organization.

The owner of the primary patent may seek to extend the effective life of their protection by filing additional patents covering new molecules, better formulations or more efficient manufacturing processes. Using these methods, they may be able to keep competitors at bay for several more years.

The patent system has many critics. Many complain that companies exploit the system by filing minor improvements as separate patents. The system is certainly slow and expensive. However, the alternatives would be for companies to rely on secrecy, like Coca-Cola does with its recipes, or to rely on state-funded research organizations to discover and develop the compounds.

Resistance was first detected in 1998 and was widespread in some major pathogens by 2000. The first patents expired from 2012 but despite all these issues, azoxystrobin and BASF's best seller, the QoI pyraclostrobin, remain the two largest selling fungicides due to careful stewardship.

Although companies are reluctant to publicize their economic thresholds, a projected return on investment of $200 million of sales per annum at product maturity may be required to support the development of a pesticide. Furthermore, using that as a measure of commercial acceptability, together with the assumption that even exceptionally good new products will capture only 25–33% of an existing market, it is possible to identify specific disease and crop targets for fungicides. On the basis of a threshold of $200 million sales annually and accepting that the industry aim is to produce market leaders, targets would have to possess a current or projected value of between $800 million and $1000 million of sales to merit inclusion not only in the development process for a new product, but also at the level of research. Of course, targets of lesser value may be considered, depending upon the evaluation of investment risk. For example, the development of a biological fungicide (see 'History' section in Chapter 6, this volume) may be cheaper than that of a synthetic, and in that case smaller markets may become commercially attractive. However, it is important to note that despite the advances in unravelling the biochemical, physical and biological bases of fungicide activity, the discovery process is still serendipitous, and it is more likely that products are made on the basis of 'develop what you discover' rather than through a strictly targeted approach.

The Global Fungicides Market

At about 26% of the total agrochemicals market, global fungicide sales are estimated to be $15.1 billion, including seed treatments (2017 figure). This compares with the size of the total human pharmaceutical market which stands at $1200 billion, of which $11.3 billion is spent combating fungal infections of humans and animals. In the pharmaceutical market compounds directed against fungi are called antifungals, avoiding confusions with the agricultural fungicides. Within the crop protection sector, herbicides are the largest component with about 40% of sales, followed by insecticides (*c.*30%) with the balance being made up of growth regulators, fumigants and miscellaneous products.

Figures from the USA indicate that 84% of fungicide use is in agriculture, with 13% in industry, commerce and government, and 3% used in the home and garden market (US Environmental Protection Agency, 2017). The two

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best-selling fungicides are the QoIs azoxystrobin and pyraclostrobin dating from 1996 and 2000 with $1000 million and $700 million in annual sales, respectively. The next three biggest fungicides are also among the oldest, with mancozeb, chlorothalonil and various copper-based products having sales of $600 million, $535 million and $355 million, respectively (MarketWatch, 2021).

Over 200 different fungicide AIs have been marketed starting with copper in the 19th century (Table 3.2) (the figure does not include BCAs; see Table 6.1 in Chapter 6, this volume). The first half of the 20th century was dominated by contact fungicides with multiple sites of action (Morton and Staub, 2008). These continued to be discovered and released up until the 2000s but research in this area seems to have all but ceased. Systemic fungicides were released after 1960. Major landmarks were the release of MBCs (B1, e.g. thiabendazole and benomyl) in 1961 and 1970 respectively, of triazoles (ergosterol biosynthesis inhibitors, G1) starting with triadimefon in 1976, of QoI fungicides (C3) starting with azoxystrobin in 1992 and of second-generation SDHIs (C2) starting with boscalid and bixafen in 2006. Contrary to the rather pessimistic view often heard at industry meetings, the rate of release of new fungicides has remained rather

Table 3.2. Fungicide introductions. (Data from Oliver and Hewitt, 2014, updated by the authors.)

Antiquity–1959

Sulfur, copper, copper oxychloride, copper sulfate, cuprous oxide, biphenyl, nabam, thiram, tecnazene, zineb, captan, folpet, blasticidin, anilazine, dodine, ferbam, metiram, fentin, quintozene

1960–1969

Fenaminosulf, binapacryl, dinobuton, dicloran, chinomethionat, ziram, thiabendazole, mancozeb, dithianon, piperalin, dichlofluanid, kasugamycin, propineb, oxycarboxin, edifenphos, ditalimfos, iprobenos, chloroneb, dimethirimol, ethirimol, fuberidazole, dodemorph, guazatine, hymexazol, carboxin, tridemorph

1970–1979

Benomyl, tricyclazole, fenarimol, imazilil, prochloraz, chlorothalonil, fosetyl-al, methfuroxam, propamocarb, fenfuram, pyrazophos, triforine, polyoxin, cymoxanil, thiophanate (ethyl), thiophanate-methyl, phthalide (fthalide), tolylfluanid, etridiazole. validamycin, furconazole, carbendazim, benodanil, bupirimate, isoprothiolane, prothiocarb, probenazole, procymidone, vinclozolin, triadimefon, triadimenol, fluoroimide, metalaxyl, etaconazole

1980–1989

Octhilinone, chlozolinate, nuarimol, propiconazole, bitertanol, mancopper, mepronil, cyprofuram, tolclofos-methyl, azaconazole, flutolanil, hexaconazole, myclobutanil, triflumizole, fenpropidin, flusulfamide, diniconazole, fenpiclonil, diclomezine, cyproconazole, flutriafol, penconazole, fenpropimorph, iminoctadine, oxadixyl, pencycuron, pyrifenox, flusilazole, maneb, diethofencarb, difenoconazole

1990–1999

Tebuconazole, carpropamid, acibenzolar-*S*-methyl, enoxastrobin, spiroxamine, copper octanoate, famoxadone, metominostrobin, fluazinam, bromoconazole, pefurazoate, tetraconazole, pyroquilon, triazoxide, ofurace, azoxystrobin, fenbuconazole, ampropylfos, ferimzone, fludioxinil, epoxiconazole, triticonazole, pyributicarb, dimethomorph, cyprodinil, imibenconazole, ipconazole, metconazole, flumorph, valifenalate, kresoxim-methyl, mepanipyrim, fluquinconazole, metalaxyl-M, furametpyr, quinoxyfen, pyrimethanil, fenhexamid, ethaboxam, trifloxystrobin, silthiofam, iprovalicarb, mandipropamid

2000–2009

Benthiavalicarb, proquinazid, tiadinil, furalaxyl-M, fluopicolide, metrafenone, bixafen, boscalid, orysastrobin, amisulbrom, dinocap, pyraclostrobin, oxpoconazole, diclocymet, fenoxanil, tebufloquin, zoxamide, fenamidone, picoxystrobin, cyazofamid, simeconazole, fluoxastrobin, prothioconazole, cyflufenamid, benalaxyl-M, diflumetorim, penthiopyrad, dimoxystrobin, iprodione, meptyldinocap, isotianil, isopyrazam, sedaxane, pyribencarb, flutianil, benalaxyl, pyrametostrobin, ametoctradin, dimethachlone

2010–date

Pyraoxystrobin, pyrisoxazole, pydiflumetofen, mefentrifluconazole, isoflucypram, coumethoxystrobin, coumoxystrobin, fluxapyroxad, pyriofenone, penflufen, fenaminstrobin, flufenoxystrobin, triclopyricarb, fenpyrazamine, benzovindiflupyr, fluopyram, mandestrobin, oxathiapiprolin, phenamacril, isofetamid, metyltetraprole, fenpicoxamid, tolprocarb, picarbutrazox

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constant at about 30 per decade for 60 years (Fig. 3.1). The rate of discovery of new MOAs remained at about two every 3 years but has recently shown a worrying trend to slow markedly. While the rate of release of new products has remained reasonably robust, the cost of developing new fungicides has risen sharply.

In the early phase of the development and use of modern fungicides, the growth of the fungicide market was slow compared with that of the more established herbicide and insecticide sectors. From about 1970, the potential of fungicides to protect both product quality and quantity became widely recognized and demand increased, stimulating an annual sales growth rate of 3–5% in the UK, for example. The increasing reliance placed by farmers on fungicides is illustrated by the steady increase in the area treated with fungicides, but the declining weight of product used (Fig. 3.2). The increase in efficacy has been due the development of systemic fungicides which typically are active in the parts per million range. Average application rates of all fungicides were over 1 kg/ha in the 1950s and have since declined to 200 g/ha (Fig. 3.3).

The Western European temperate cereal and vine industry was traditionally the largest fungicide market, but other regions and crops are fast catching up. Europe still has 30% of world sales, the Asia-Pacific region and Latin America each have about 25%, and North America has 15%. In Asia and the New World, fungicide sales

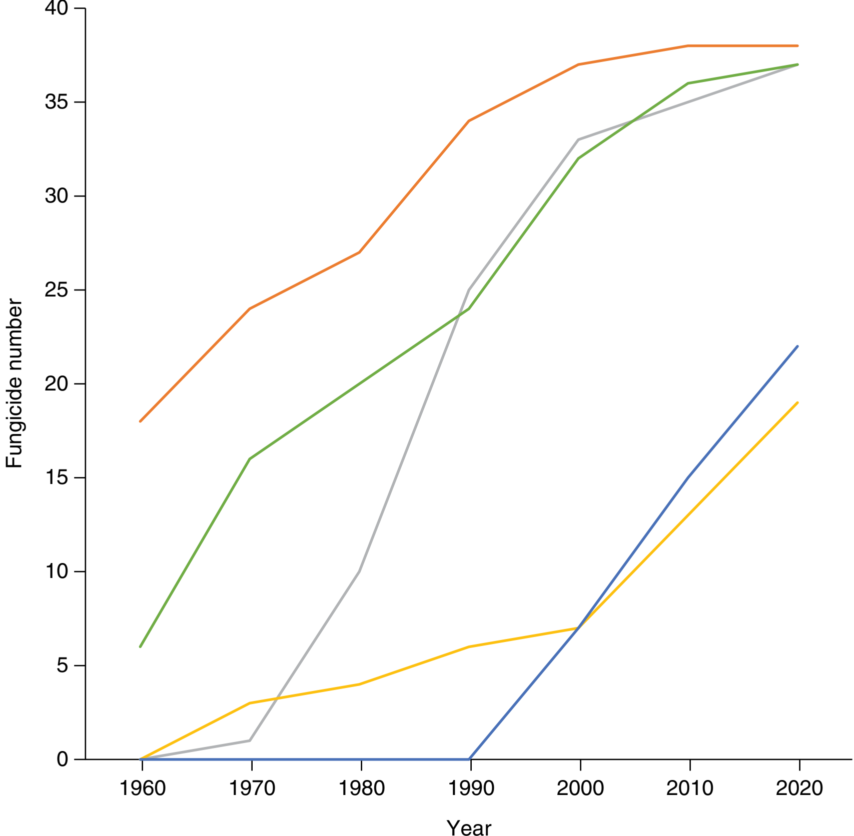

Fig. 3.1. The number of non-systemic (——), SBI (G1; ——), SDHI (C2; ——) and QoI (C3; ——) fungicides, as well as different MOAs (——), released since 1960. The total number of fungicides released from 1960 is steadily accumulating but the first signs of a slowdown in the rate of new releases may be apparent. SBI, sterol biosynthesis inhibitor; SDHI, succinate dehydrogenase inhibitor; QoI, quinone outside inhibitor; MOA, mode of action. (Authors' own data.)

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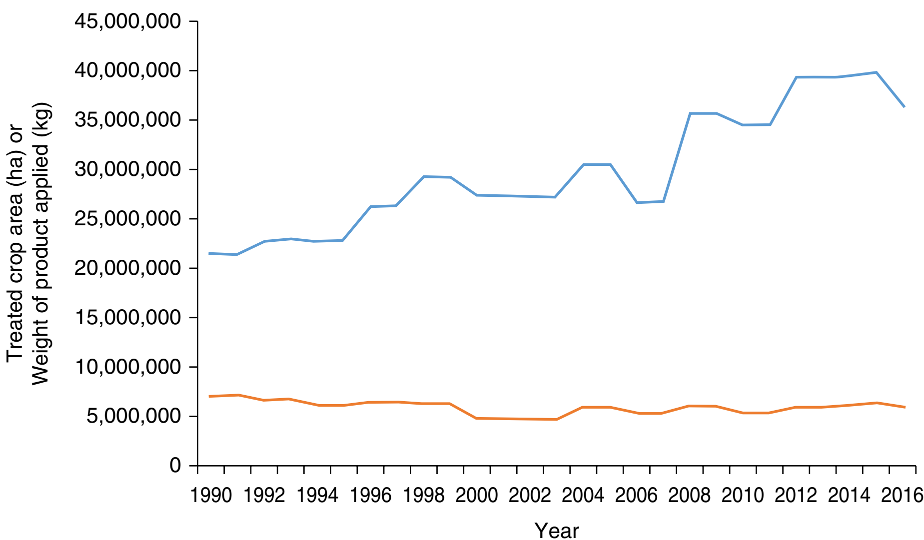

Fig. 3.2. The UK fungicide market, showing an increasing area of treated crops (——) and a slowly declining weight of product applied (——) from 1990 to 2016. The total cropped area of the UK is about 6 million ha, so on average each field is receiving 6 applications of fungicides per year. Despite that, the weight of product applied is declining reflecting the use of more potent products that allow lower doses to be used. (Data from PUSSTATS.)

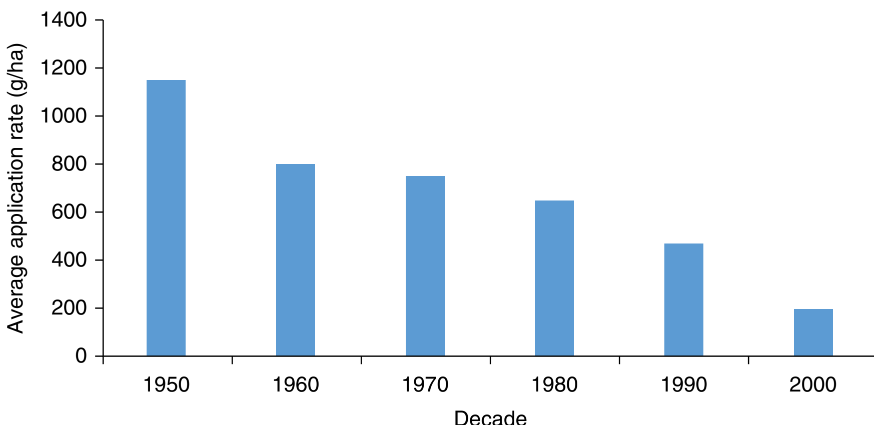

Fig. 3.3. Average fungicide application rates have declined substantially from the 1950s to the 2000s as product efficacy has risen. (Data from Phillips McDougall, 2018.)

were restricted due to low crop values or the presence of yield-limiting factors other than disease, such as water deficiency. However, since the early 1990s fungicide sales have grown at over 5% per annum in those regions, in response to increased usage in South-East Asia on rice and in Latin America on high-value crops such as bananas.

More recently, and according to the incomplete data from the Food and Agriculture Organization of the United Nations (FAO) (Table 3.3), many low- and middle-income countries such as Brazil, Mexico, Colombia and Bangladesh have become major fungicide users (Schreinemachers and Tipraqsa, 2012).

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Table 3.3. Major fungicide users by weight, 2012. (Data from FAOSTAT.)

Country	Tonnes
Brazil	37,381
Italy	36,457
France	27,355
Spain	26,798
Mexico	24,776
USA	24,040
Japan	23,528
Colombia	22,387
Turkey	18,124
Ukraine	11,754
Bangladesh	10,618
Germany	8,774
Portugal	8,499
Canada	7,547

Fungicide sales by mode of action

Three fungicide classes currently dominate global sales (Table 3.4), with DMIs, QoIs and SDHIs making up over 60% of sales. The DMI group has been the mainstay of foliar disease protection for 50 years, whereas the QoIs have established their market position only since 2000. Resistance to both these fungicide classes has become a major factor in the last 15 years. SDHI fungicides were first released in 1966 but had a limited impact. Many new SDHIs have been released since 2003 and they have since rapidly assumed a significant market share up from 3.5% in 2012 to 11% in 2016.

There are a large number of older fungicides that have maintained sales in various niche markets and as mixing partners with the three main MOA classes. A good safety record is essential as many fungicides have been deregistered. Many older contact fungicides with multi-site MOAs retain large market shares after many decades of use. This is a testament to the efficacy of their action, their safety record and the economic benefit they give to the grower. The strong sales of the sole chloronitrile, chlorothalonil, can be attributed to its value as a mixing partner with QoI, DMI and SDHI fungicides although it was deregulated in Europe in 2020. Sales of inorganic fungicides based on copper and sulfur are showing strong growth, rising from 82 kt in 2012 to 94 kt in 2017 partly to service the growing market for crop protection

Table 3.4. Market share of different fungicide groups. (Data from FAOSTAT.)

		Market share (%)	
Fungicide group	Code	2012	2016
Demethylation inhibitors (DMIs)	G1	29	27
Quinone outside inhibitors (QoIs)	C3	22	22
Succinate dehydrogenase inhibitors (SDHIs)	C2	3.5	11
Others		45.5	41

in the Biological/Organic sector (FAOSTAT) (see ‘History’ section in Chapter 6, this volume).

Global fungicides market by crop

Fungicide manufacturers focus resources on the research and development of new products that fit the most valuable markets. In terms of crops, vegetables, temperate cereals, rice, grapevine, potato, soybean and pome fruit dominate the global fungicides market, representing nearly 85% of the global sales value in 2005. These ratios have proved to be fairly constant but there has been a large increase in value of the soybean market, which has increased from 1.1% in 1990 to 8.3% in 2005 and to 14.8% by 2016, with a corresponding relative decline in the cereals market to 19% and the ‘other fruit and vegetables’ share to 15.8% (Bryson and Brix, 2019).

Large fungicide markets are attractive not only because of their size, but also because they utilize long-established and well-understood technologies and present clear challenges for new-generation compounds. Absolute value, however, has to be balanced against the diversity of targets within a particular market, an assessment of current and potential competition, the level of technology required to succeed in that market and a view to future commercial and technical trends.

Only vegetables ($1.72 billion), temperate cereals ($1.20 billion), rice ($740 million), grapevine ($700 million) and pome fruit ($320 million) can be considered as potentially viable commercial targets for investment in the

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discovery and development of new fungicidal products. The vegetable market is highly segmented, comprising many crops and a broad spectrum of pathogens. Accordingly, the registration of new products into this market is expensive and as a general target, vegetables do not offer a viable return on investment. Hence, fungicides sold into the vegetable market are always well established for use against pathogens in commercially more important sectors such as cereals. An exception is potatoes where fungicide use has become very intense in Europe. The inadvertent introduction of the *Phytophthora infestans* second mating type into Europe in the 1980s allowed the organism to circumvent numerous disease resistance genes that were previously effective (Gisi and Sierotzki, 2015). As a result the fungicide companies have introduced oxathiapiprolin, valifenalate, ametoctradin and fluazinam to complement the established metalaxyl family of fungicides.

Leading Fungicide Manufacturers

The rising costs of the development of new fungicides and the maintenance of existing products due to increased regulatory pressures have encouraged the industry to consolidate. Consequently, companies have become increasingly international and, through merger, acquisition and considerable good luck in the discovery and development of key products, a few have emerged to dominate the market. A second driver in the development of the fungicide industry has been the need to focus on the specific needs of the crop protection market. The pioneer companies in this area were general chemical companies. The companies synthesized a relatively small number of chemical leads and then tested them for utility not only in crop protection, but also for pharmaceutical, cosmetic, domestic and industrial uses. These sectors gradually diverged so that separate synthesis streams were developed for each market. The bottleneck shifted from chemical synthesis to biological testing and so many of the companies split into smaller more specialized entities. More recently, these specialized crop protection companies expanded their activities into related agricultural areas especially by developing activities in plant breeding and seed production.

Two decades ago, there were ten major companies, but currently only four can be considered full-scale fungicide discovery and production companies. These are Syngenta (2018 sales of $3.1 billion), Bayer CropScience (incorporating Monsanto) (2017 sales of $2.9 billion), BASF (2015 sales of $2.5 billion) and Corteva (formerly Dupont and Dow) (sales not reported). Syngenta, which was formed from a merger of Zeneca and Novartis in 2000, merged with Chemchina, a large state-owned Chinese company, in 2018. Sandoz and CIBA were previously acquired by Novartis and Zeneca was derived from ICI. Bayer CropScience was formed from the fusion with Aventis in 2002. Earlier predecessors were AgrEvo and Rhône-Poulenc. Corteva was formed as a spin-out of the merger of Dow and DuPont in 2018. Syngenta, Bayer and Corteva are all focused entirely on the agriculture market, producing not just agrochemicals but also seeds. BASF is unique is remaining a broad-based chemical company of which agrichemicals are only a small part.

Another group of companies specializes in manufacturing and distributing off-patent (or 'generic') compounds. They thus avoid the huge cost and risk of fungicide discovery and development. They do incur the costs of registration in smaller markets. On the other hand, they survive only if they undercut the original patent holder so their profit margins will always be limited. There are many generics companies in the fungicide area. Prominent examples include Nufarm (2019 sales: $410 million), Helena (2019 sales: $221.2 million) and FMC (2019 sales: $75 million).

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4

Fungicide Discovery

Key Points

- Strategies to discover new fungicides focus on:
  - experimentally tractable pathogens;
  - diseases with the greatest market potential;
  - new and emergent diseases; and
  - replacing MOAs suffering from resistance.
- 'Leads' are active compounds that can be modified for useful field performance.
- Screens for leads use *in planta*, *in vivo* and high-throughput strategies.
- Sources of fungicide leads include random compound libraries, natural products, combinatorial chemistry, compounds designed to inhibit specific enzymes and compounds with optimized physicochemical properties.

Target Selection

Market size

Each agrochemical company has its own commercial strategy with respect to target definition, but all adopt the same general process, known as screening, to identify product candidates. While the discovery of fungicides necessarily includes aspects of biochemistry, synthetic chemistry and formulation, commercial success is founded not upon the ability of a company to deliver clever chemistry, but on the field performance of its products. The driving forces of fungicide discovery, therefore, are the determination of biological activity, novelty of the MOA and its transfer to useful field performance. The composition of the screen reflects the value placed by the company on the control of the various crop–pathogen combinations and the overall value, in terms of fungicide sales, associated with each crop.

The main factors in determining the scale of the market are the area planted to the crop, the degree of losses caused by each pathogen and the cash value of the lost yield. The total losses attributed to pathogens and pests remain stubbornly high across nearly all crops and regions. A recent review of losses to pests and pathogens of the five major arable crops (wheat, rice, maize, potato and soybean) worldwide showed that they averaged between 17 and 30% (Savary *et al*., 2019) (Table 4.1). Average losses in different parts of the world did not vary substantially despite major differences in production styles. For all five crops the dominant aetiological agents were fungi or oomycetes and thus suitable targets for fungicidal control. And in any one area, a maximum of three and sometimes just two pathogens caused more than

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Table 4.1. *Fungi* and *Oomycetes* causing more than 2% losses in any global region. (Based on data taken from Savary *et al*., 2019.)

Wheat	Rice	Maize	Potato	Soybean
PUCCRT	RHIZSO	GIBBZE	PHYTIN	SCLESC
GIBBZE	PYRIOR	COCHCA	ALTESO	PHAKPA
SEPTTR	COCHME	COLLDU		CERCKI
COCHSA	USTNVI	CERCZN		PHYTMS
PYRNTR		PUCCSO		
ERYSGT		SCPHMA		
LEPTNO				
PYRIOR				

50% of the losses. Any fungus or oomycete on this list is likely to constitute a suitable target.

These bulk commodity crops are grown on huge scales which can provide the basis for a large enough market to justify the development of a fungicidal product even though the value of these crops on a weight or area basis is limited. Thus, per annum, the market for fungicides aimed at SEPTTR on wheat is currently about $1500 million, rice PYRIOR is $600 million, soybean PHAKPA is $500 million, and potato and tomato PHYTIN is $100 million.

The next level of crops is represented by barley, sorghum and oilseed rape (canola) (Table 4.2). A disease unique to one of these crops is not likely to produce a sufficient incentive to develop a specific fungicide but would instead provide a secondary use of products developed on a first-level crop disease.

Horticultural crops are grown on much smaller areas, but the product values are typically much higher. Crops such as bananas, grapevines, pome fruits and citrus fall into this class and in addition are perennial. In contrast to annual crops, perennials are much slower to breed for disease resistance and their woody tissues can often harbour pathogen spores during the non-growing season. Both factors make non-chemical disease control methods much more difficult, so growers are more likely to be reliant on fungicides. For grapevines, three diseases PLASVI, BOTRCI and UNCINE are significant targets in many wine-growing areas, as is MYCOFI for banana production. Hence the high value of many horticultural crops combined with the extra difficulty many crops have in controlling diseases mean that they can represent key targets for fungicide development.

Table 4.2. Secondary fungicide targets. (Authors' own data.)

Barley	Sorghum	Canola
ERYSGH	COLLGR	SCLESC
PUCCHD	SCPHMA	RHIZSO
PYRNTE	GIBBZE	PYTHSP
RAMUCC	PYTHSP	LEPTMA
RHYNSE		PYRPBR
GIBBZE		

New and re-emergent diseases

The incessant demand for more food means that crops are being grown in areas that previously were used for other purposes. South America has seen a large expansion in the areas sown to soybean and wheat. Soybean cultivation has provided the perfect setting for the invasion and expansion of soybean rust caused by PHAKPA. The wheat area is generally warmer and more humid than in traditional wheat-growing areas and this seems to be the reason why wheat blast, caused by a close relative of the rice pathogen PYRIOR, has taken off. Both crops are now considered to be major fungicide targets.

Fungicide resistance

Fungicide resistance has become one of the dominant factors in target choice. Pathogens differ in their propensity to develop resistance and the pathogens that typically develop resistance first are the powdery mildews and BOTRCI (for details see 'Pathogen risk factors; fecundity; latent period; sexual reproduction' section in Chapter 11, this volume). For this reason, it is

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still economic to develop narrow-spectrum compounds that are specific for these pathogens. Recent examples for powdery mildews include quinoxyfen, metrafenone, bupirimate, proquinazid, spiroxamine and cyflufenamid; and, for BOTRCI, fenhexamid, fludioxonil and iprodione.

The importance of fungicide resistance has placed a premium on compounds that either will not develop resistance or would protect high-risk compounds from developing resistance. Indeed, the design of compounds that would be immune from resistance can be said to be the current Holy Grail of the industry. The value of compounds that protect high-risk compounds explains the increased market share of chlorothalonil, used as a mixing partner for QoI fungicides, although it has recently been banned in Europe and replaced in that role by folpet.

The differential ability of pathogens to develop fungicide resistance appears to be one of the major reasons which explains the current prominence of different diseases. Twenty years ago, the dominant diseases of barley were leaf rust, powdery mildew and scald. Each of these diseases can be well controlled by genetic methods in combination with existing fungicides. Currently the major diseases of barley include RAMUCC and PYRNTE. Both these pathogens are difficult to control by genetics and appear to be adept at developing fungicide resistance. Hence both can now be considered important secondary targets. A similar scenario applies to oilseed rape (canola). Blackleg (LEPTMA) was the major disease worldwide, with major efforts being aimed at maintaining genetic control. Recently light leaf spot (PYRPBR) has emerged as the major threat as, being polycyclic, it can circumvent both genetics and chemistry rapidly.

New modes of action

The development of resistance in pathogen populations reduces or eliminates the efficacy not only of the fungicide in the test, but also of all others that share its MOA. As only a handful of MOAs is available, resistance is a major threat not just to fungicide company profits but also to global food production. Hence, fungicide companies are not merely seeking new fungicides that can be patented and marketed but entirely new MOAs. This realization has altered the way fungicide discovery takes place. Paradoxically, companies are seeking compounds with unknown MOAs. This has placed a premium on the imagination and inventiveness of the researchers.

Screening for Fungicide Leads

A screen is a stepwise series of tests that challenge a candidate pesticide with increasingly difficult biochemical and/or biological hurdles. The steps can be aspects of MOA, application rate, spectrum, phytotoxicity or redistribution in the crop, but essentially need only to include those attributes that affect the practical use of the candidate fungicide by farmers and hence its commercial value. In principle, the term 'screening' can encompass all steps in the biology of pesticide discovery and development up to product status, but it is usually understood to describe only laboratory and glasshouse tests. A key property of a fungicide is the concentration needed to control a disease; see Box 4.1 for a discussion of the parameters used to describe quantitatively the potency of a fungicide.

The design of fungicide screens

Screens used by fungicide companies can be divided into three broad classes referred to as 'high-throughput', *in vivo* and *in planta* (Table 4.4). These types of screens represent the dilemma of choosing between cheap and easy tests on huge numbers of compounds, but which only rarely lead to a useful product, versus slow and expensive tests of only a few compounds that individually have a much better chance of being ultimately useful.

In vivo *screens*

In the fungicide industry, *in vivo* refers to the growth of a fungus away from a plant. For non-obligate species, it is normally a simple matter to grow them in an agar plate or microtitre well, add aliquots of test compounds and measure the degree of growth inhibition. *In vivo* tests use much less compound than *in planta* tests. When non-obligate filamentous fungi are inoculated

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Box 4.1. Measuring and describing fungicide potency: EC_{50}s, IC_{50}s and MICs.

A key property of a fungicide is its ability to control a disease. However, it is surprisingly complex to define the potency of a fungicide in a simple and quantitative manner. This has led to the definition of at least three key parameters – the EC_{50}, the IC_{50} and the MIC. These stand for the 'concentration of the fungicide that gives 50% effective control', the 'concentration of the fungicide that gives 50% inhibition' and the 'minimum inhibitory concentration', respectively. Each of these is used to define a single parameter that describes how effective a fungicide is.

All these tests require quantitative input data, and these can be the radial growth rate of a fungus grown on a plate or in a microtitre dish, an enzyme activity, binding of a fungicide to a target, the amount of disease on a plant or the yield of infected plants. The parameter is then measured at a range of concentrations of the fungicide. Graphs of the parameter and the fungicide concentration define the 'dose–response curve'. A typical and somewhat idealized curve is shown in Fig. 4.1. At low concentrations, there is no impact while at high concentrations the fungus is completely killed. The focus of the EC_{50} or IC_{50} is to find the concentration at which the fungus is inhibited to 50%. These concepts come from pharmacology. In the fungicide world we are almost exclusively concerned with inhibition. Pharmacologists call this antagonism, but they also often deal with drugs that induce activity and thus are called agonists. The EC_{50} parameter can be used to describe both agonists and antagonists, whereas IC_{50} works only for the latter. Hence the fungicide industry tends to use IC_{50} even though EC_{50} is in much of the literature.

The dose–response curve ideally has horizontal sections at both very low and very high concentrations. To define the IC_{50}, a third horizontal line 50% of the way between the minimum and maximum lines is drawn on the graph. The accuracy of the final IC_{50} is critically dependent on how accurately the position of the 50% line can be defined. A vertical line is drawn from where the 50% line crosses the dose–response curve, defining the IC_{50} concentration.

IC_{50} graphs use up a large amount of the test compound. It is also possible that the fungicide cannot be readily dissolved at the highest concentrations needed. For these reasons often it is sufficient to define an MIC, a minimum inhibitory concentration. In an MIC test a standardized series of concentrations is placed in a growth medium. After inoculation and growth of the pathogen, it is conceptually simple to note down the lowest concentration that has no growth. This is not as accurate as an IC_{50} and it sometimes can be difficult to precisely define the point of zero growth, but provided the test is always done in the

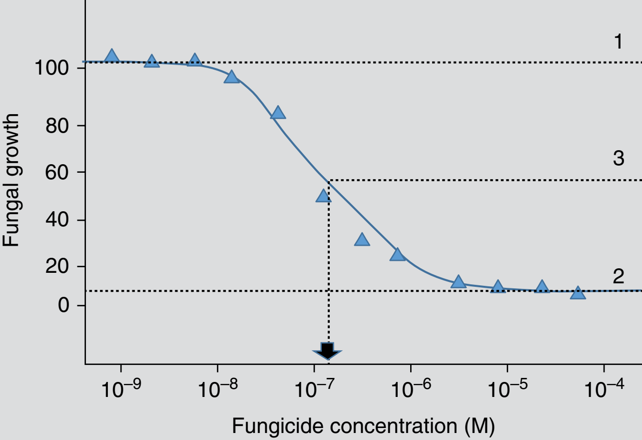

Fig. 4.1. Determination of half-maximal inhibitory concentration (IC_{50}) values. The amount of fungal growth is plotted against the fungicide concentration over a wide range. Horizontal lines are drawn through points corresponding to maximum (1) and minimum (2) growth. The point at which the third line (3), drawn half-way between 1 and 2, crosses the sigmoid line of growth gives the concentration of 50% inhibition.

Continued

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Box 4.1. Continued.

same way, the results will be useful. To further complicate matters, some reports focus on the EC or IC dose that inhibits growth by 20, 80 or 90%, referred to as the EC_{20} or IC_{20}, etc. It pays to carefully read the report to be certain what is being determined and how the data were analysed.

Fungicide concentrations can be expressed as parts per million (ppm) or even parts per billion (ppb). These are equivalent to mg/l or μg/l. As fungicides typically have molecular weights in the 200 to 400 range, an IC_{50} of 1 ppm would be about 3.3 μM. The search for ever lower IC_{50}s is illustrated by Table 4.3. A field rate of 490 g/l equates to an IC_{50} for inhibition of cytochrome c reductase of 0.15 μM, 45 μg/l or 0.045 ppm.

Table 4.3. The increase in potency of fungicides released since 1940. (Data from Phillips McDougall, 2018, with permission.)

	Dithiocarbamates	Morpholines	Triazoles	Strobilurins	Second-generation SDHIs
Period of introduction	1943–1967	1968–2003	1976–2002	1996–2007	2003–date
Typical field rate (g/ha)	2500	590	140	490	100

SDHI, succinate dehydrogenase inhibitor.

Table 4.4. Characteristics of different types of fungicide screen. (Authors' own data.)

Type of screen	Amount of test chemical needed	Indicative number of chemicals that can be tested per annum
High-throughput tests	Less than 1 mg	100,000
in vivo tests	Micrograms	10,000
In planta tests		
Detached leaf tests	Milligrams	1,000
Glasshouse, whole plant sprays	Grams	500
Outdoor plot trials	100 g	100
Field trials	Kilograms	10

into the centre of an agar plate, they grow outwards at rates typically between 1 and 10 mm/day. If the plate contains a test compound, the reduction in radial growth rate caused by the compound can be easily measured (Fig. 4.2). Multiple compounds can be added to different sectors of a plate to increase the number of tests either in wells cut in the agar or via small paper discs soaked in the compound. Agar plates are large and unwieldy, so companies often prefer to use microtitre plates that have 96 wells in an 8 × 12 array. The growth of the fungus can be measured by assaying turbidity (light scattering) in the well using automated equipment. An 8 × 12 plate can be used to test 12 compounds at eight different concentrations, or 24 compounds at four different concentrations. Microtitre plates are also suitable for determining the potency of compounds to inhibit growth of fungi with a yeast-like growth habit – that is, growth by cell division (Fig 4.3b). Apart from SACCCE, SEPTTR also grows as a yeast under *in vivo* conditions. Another method suitable for yeasts is to drop serial dilutions of cells on to a plate. At higher compound concentrations, fewer cells survive to form a colony. The potency of a compound can be estimated by dropping serial cell dilutions on to a standard concentration, e.g. 1 ppm. The lowest number of cells that survive to give a countable number of colonies can easily be determined (Fig 4.3a).

In vivo tests tend to generate many false positives and even a few false negative results, and hence are treated with some suspicion. The false positive results occur when a compound that inhibits growth in the plate assay fails to inhibit growth in the plant. There are many reasons why this might be the

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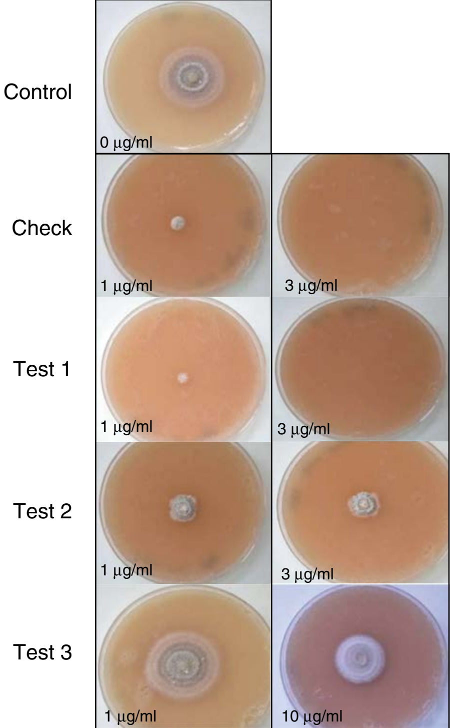

Fig. 4.2. Radial growth assays of LEPTNO. Each plate contains a nutrient agar medium amended at two concentrations with solvent (control), check (current fungicide) or test compounds. The plates are inoculated with spores or a mycelial plug in the centre and allowed to grow for 2–7 days. The average radius of growth is measured.

case. The main ones are that the compound may not be translocated in the plant or may be metabolized into an inactive form by the plant. Hence all *in vivo* tests must be followed up with *in planta* studies.

Conversely, there are a few cases where an *in vivo* test would give false negative results. Examples would be compounds such as ASM and probenazole that work by activating plant defence. Discovery of such compounds requires a different and specific strategy.

In vivo tests are only available for pathogens that can be grown axenically – away from a plant. Several of the most important pathogens cannot be grown on artificial media. Such obligate pathogens include all the rust species (PUCCXX, PHAKPA) and all the powdery mildews (ERYSGH/T, UNCINE), downy mildews (PLASVI) and a few others. For these species, there are no substitutes for *in planta* tests.

High-throughput tests

High-throughput tests encompass a range of tests with the common factor of being faster than an *in vivo* test. They are very varied in design and include cell-free enzyme assays, microbial strain growth or microbes expressing a reporter gene. The goal was to screen very large numbers of compounds with an assay designed to reflect some essential function of the pathogen. However, the advantages of high-throughput were soon seen to be outweighed by the disadvantages; very few compounds that were active in the high-throughput test proved to be useful as leads. A compounding paradox was that it was too easy to find compounds that were active in the high-throughput test. Further tests using *in vivo* and *in planta* assays were consuming inordinate amounts of time in company laboratories and leading to few useful leads. Hence this approach has largely been abandoned.

An example of a high-throughput test is the yeast YUG37:*erg11* expression system (Cools *et al.*, 2010). The target of the G1 sterol-biosynthesis DMI class of fungicides is the enzyme CYP51. In yeast the equivalent protein is called ERG11, encoded by *ERG11*. DMI fungicides had been introduced in the 1970s and given excellent performance for the next 25 years. A decline in the performance of DMI fungicide was noticed. Sequencing of the *Cyp51* target gene from various species showed that there were various mutations, but there were no ways to definitively link them with the resistance phenotype using the technology available for pathogenic fungi at the time.

Genetic technologies were developed for yeast much earlier than for filamentous fungi in general and pathogens in particular. The concept of the yeast expression system was to replace the endogenous *ERG11* gene in yeast with the *Cyp51* from the pathogenic fungus. The pathogen gene could either be the wild-type version or one from a putatively resistant gene. The pathogen gene is then expressed in yeast and the sensitivity of the strain to DMI fungicides can easily be determined (Fig. 4.3). To focus attention on the pathogen gene, it is necessary to switch off the yeast *ERG11* and this is

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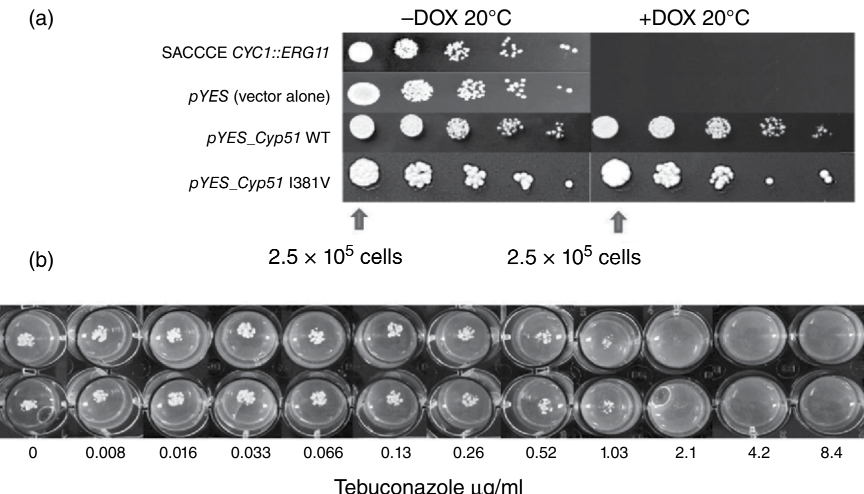

Fig. 4.3. Using the yeast *ERG11*/*CYP51* expression system. (a) Growth of the SACCCE strains with and without doxycycline (DOX). The first two rows show that the host strain *CYC1::ERG11* cannot grow when doxycycline is added as this represses the promoter driving expression of the endogenous yeast *ERG11* gene. The next two rows show strains where two gene variants of a pathogen *Cyp51* gene are expressed, showing that the fungal gene can replace (complement) the yeast gene. 250,000 cells were added to the first column and tenfold less on each subsequent column. Individual colonies can be scored in the columns where 250 and fewer cells were pipetted. (b) Growth of the *pYes_Cyp51* wild-type (WT) strain in microtitre wells with increasing concentrations of tebuconazole. The turbidity of the wells can be used to measure the IC_{50}. (From F. Lopez-Ruiz with permission.)

done by placing the yeast gene under the control of a promoter (*CYC1*) that is repressed by doxycycline. The yeast strain is grown in the absence of doxycycline to ensure its viability. However, addition of the drug forces the strain to rely on the pathogen gene. Such reporter gene strains can be used to screen novel compounds for activity.

Mode-of-action screens

Assays with the features of high-throughput screens are used to determine the MOA. Fungicide companies are particularly keen to discover compounds with new MOAs as they are very likely to be novel and therefore hold out the promise that the company could develop a dominant position over a whole class of compounds. Furthermore, as there are so many problems with fungicide resistance affecting all major groups of fungicide, a new MOA is likely to have a large market both replacing and protecting fungicides affected by resistance.

Hence companies have developed high-throughput assays that report whether a compound has each of the known MOAs. If an active compound scores negative in each of the tests, the hunt for the new MOA is initiated. The exact methods behind these assays are closely guarded secrets.

In planta *screens*

In planta screens are more time-consuming and expensive than the *in vivo* tests but also more predictive of final success. An *in planta* test is one where the pathogen undergoes its infective life cycle on living plant tissue. The plant tissue may be a seedling or explant grown in soil for several weeks in a glasshouse or growth chamber. At an appropriate stage, the pathogen is inoculated and the plant is incubated so as to promote disease.

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The test chemicals may be applied *before* the pathogen to screen for *preventive* activity or *after* to screen for *curative* activity. The amount of disease is scored some days or weeks later and compared with that produced by the pathogen alone. In most cases expression of good disease symptoms requires incubation of the infected plants in high humidity and controlled temperatures, so-called misting chambers. Performing these *in planta* tests is a demanding process requiring highly skilled staff and extensive and expensive facilities. It explains the many hectares of glasshouses found around the grounds of all fungicide companies. Such *in planta* tests also require relatively large amounts of the test compounds – at least a few milligrams and possibly several grams (Fig. 4.4).

For all these reasons, primary compound screening tests typically use some sort of detached leaf assay. Leaf discs or short sections as small as 5 mm are cut out, often with specialized machinery but also by hand, and then placed on a special agar or liquid medium. The medium contains a cocktail of compounds proven to maintain the healthy life of the leaf piece, long enough for the pathogen to complete its life cycle. The pathogen is then dusted or pipetted on to the leaf pieces. The test compounds may be sprayed on the leaf pieces or may be incorporated in the bathing medium. In the latter case, the companies would need to be aware of the potential for the compound to translocate into the leaf piece and thus come into contact with the pathogen. Finally, after an appropriate period the degree of infection is assessed either by eye or by some sort of computerized image analysis. The infection level is normally converted to a per cent disease control parameter.

All *in planta* screens have the advantage that they tell the researcher whether the compound

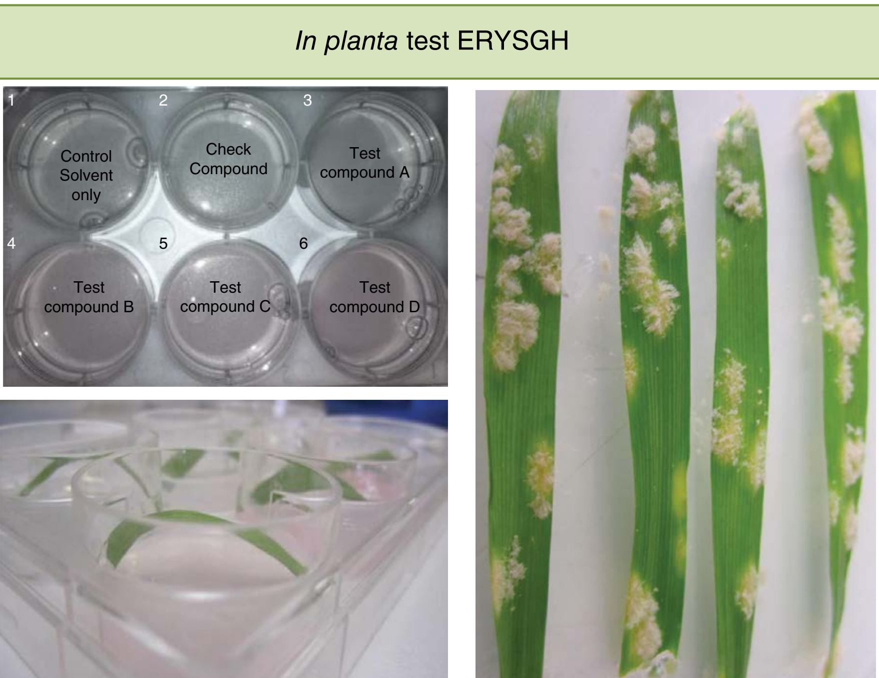

Fig. 4.4. *In planta* test of compounds against ERYSGH. Leaves of a susceptible barley cultivar are excised and placed on an agar suspension containing supplements that inhibit senescence. Each well contains a different compound, but with the same solvent: well 1 has no compound and is a positive control; well 2 has a standard check compound; wells 3–6 have four test compounds (top left). Spores are dropped on to the leaves and the plates are sealed and incubated in moderate light (bottom left). After 1 week the infections are scored (right).

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is toxic to plants, exhibiting so-called phytotoxicity. But even if a compound is safe to plants and inhibits the disease *in planta*, it may not be suitable as a fungicide. Many will prove to be toxic to non-target organisms or may have insufficient stability or rainfastness to work in the field.

If compounds prove successful in the various laboratory and glasshouse trials, they may be further tested in plot trials on plants grown in realistic field conditions. Crops are grown outdoors to adulthood. The method of inoculation will vary depending on the pathogen. In some cases, natural infection will occur without further action. In others it may be sufficient to spread infected stubble around the base of the plants or merely to regularly irrigate the plants with overhead misters. In most cases, pathogen spores will need to be propagated in the laboratory and then sprayed on to the plants. Disease symptoms can then be scored some days or weeks later. The advantage of the outdoor plot trials is that they replicate the conditions of temperature, sunlight and rainfall that will be experienced by the crop in a normal farmer's field. The test compound will therefore need to demonstrate adequate stability to ultraviolet (UV) light and rainfastness – the ability to withstand being washed away by typical rainfall. These tests are subject to the vagaries of the weather, and of contamination by other pathogens. Their high expense is only justified if there is already a good deal of confidence that the compound will lead to a successful product.

Primary target organisms

Fungicide companies have a set of primary target pathogens against which new compounds are screened. The names of the primary targets are commercial secrets, but one would guess the list shown in Table 4.5. Companies not only focus on the pathogens with the biggest potential market sizes but will also pay attention to taxonomy. A lead compound that had activity against more than one of the major taxonomic groups would attract extra attention. QoI fungicides are exceptional and owe their large market size to having activity against basidiomycete, ascomycete and oomycete pathogens.

Another factor when choosing primary target organisms is the ease with which they can be tested in a laboratory setting. Pathogens that can be grown in defined artificial media are much more economical to test than ones that must be tested on living plant tissue. Fast-growing fungi such as SEPTTR and BOTRCI are favoured for that reason over VENTIN and MYCOFI. It is, however, an unfortunate fact that many of the priority targets are obligate pathogens which are the most difficult to handle.

In addition, some non-pathogenic fungi are widely used in fungicide discovery laboratories. These include the yeast *Saccharomyces cerevisiae* (SACCCE) and the filamentous species *Aspergillus nidulans* (ASPEND) and *Neurospora crassa* (NEUSCR). The non-pathogenic fungi have been used in fundamental science as model systems because of their ease of culture and fast life cycles.

Table 4.5. Characteristics of major fungicide test organisms. (Authors' own data.)

Code/pathogen name	Disease	Host	Taxonomy	Facultative/ obligate
SEPTTR	Septoria tritici blotch	Wheat	Ascomycete	Facultative
PYRIOR	Blast	Rice and wheat	Ascomycete	Facultative
UNCINE	Powdery mildew	Grapevine	Ascomycete	Obligate
ERYSGT/H	Powdery mildew	Wheat and barley	Ascomycete	Obligate
PUCCRT	Brown rust	Wheat	Basidiomycete	Obligate
PHYTIN	Late blight	Potato/tomato	Oomycete	Facultative
BOTRCI	Grey mould	Many but especially grape	Ascomycete	Facultative
PLASVI	Downy mildew	Vine	Oomycete	Obligate
PHAKPA	Asian soybean rust	Soybean	Basidiomycete	Obligate
VENTIN	Scab	Apple	Ascomycete	Facultative (but very slow growing)
MYCOFI	Black sigatoka	Banana	Ascomycete	Facultative (also slow growing)

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Generations of scientists have generated extensive genetic resources such as complete mutant libraries and functional genetic technologies. The first fungal genome sequences to be made publicly available were of these model system fungi (Cools and Hammond-Kosack, 2013). Yeast can be regarded as a good model for all fungi, but it lacks a filamentous phase and so would fail to detect inhibitors of chitin biosynthesis. The ability to manipulate some model system fungi (and indeed bacteria) means that a specific screen can be designed using engineered yeast strains. For these reasons, many MOA screens rely on model fungal species.

Functional genomics

The range of tools available for fungicide discovery and research has undergone a revolution in the last two decades (Cools and Hammond-Kosack, 2013). The traditional picture of a fungicide company research department is that chemists produce compounds and biologists determine whether they kill pathogens and prevent disease. Molecular biology offers much to streamline and focus this work. The goals of a fungicide company are to find compounds with high potency, broad spectrum for pathogens and little or no impact on non-target organisms starting with the host plant but including all other organisms in the environment. The first pathogen genome sequences were reported between 2000 and 2005 and cost $1 million or so to generate and analyse. Nowadays, genome sequencing of a pathogen is regarded as a trivial task costing $1000 or less. Genome sequences of host plants have been obtained and released by academic laboratories. Using these data it is possible for a company to generate lists of genes that all the pathogens possess, and which are absent or substantially altered in non-target organisms. Such genes are good targets for broad-spectrum fungicides.

Functional genomics is the term used to describe the processes needed to determine the role of each of these genes. This can then be used to select genetic targets for fungicides. A good target gene would be present in all pathogens and would also be essential for viability. Hence the list of ever-present genes can be edited to include only those genes that are essential. The range of tools needed to answer this question is only slowly being finalized for relevant pathogens (Cairns *et al.*, 2016) and studies in this area have been led by human pathogens such as *Candida albicans* and only more recently applied to phytopathogens (Chaudhari *et al.*, 2019). Thus far there are no reports of fungicide discovery using such tools, but the increasing difficulty in finding new successful leads suggest that these new methods will soon bear fruit.

Sources of fungicide leads

The term ‘lead’ is used widely in the industry and refers to the first compound that shows activity against a target fungus. The chemical structure is determined and then many variants are synthesized. These variants are also tested in the assays until the structural features associated with activity are identified.

Fungicide leads arise in five ways:

1. Random chance.
2. Combinatorial chemistry.
3. Analogue synthesis.
4. Biorational design.
5. Chemorational design.

Random screening

Traditionally, fungicide discovery uses serendipity which, at the most fundamental, relies on the laws of chance for success. If enough compounds are supplied and tested, provided a screen is constructed to meet the required commercial targets, a product is guaranteed. In this system, compounds submitted for screening are chosen in the absence of any prior knowledge of structure–activity relationships or novelty of chemistry. The chemistry of many compounds may be unknown or not divulged, being obtained from third parties under a confidentiality agreement. They may also be purchased or synthesized in-house, either as end products of speculative programmes or as intermediates.

An important source of test compounds is natural products. Certain academic laboratories and specialized lead discovery companies focus on the identification of various types of organism from which are extracted the products of their secondary metabolisms. Such metabolites will vary depending on the culture condition.

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A classic success story for the natural product route is the strobilurins. The original set of compounds was extracted from the fungi *Strobilurus tenacellus* and *Oudemansiella mucida* (Anke *et al*., 1984; Sauter *et al*., 1999). Over a 20-year period the structure of the compounds was determined, and their activity tested. Despite being very active and with a very good spectrum, they proved too unstable for use in the field and were only released after extensive modifications.

Although the chance of finding a compound is vanishingly small, random screening, used as a lead-generating activity rather than a process to identify products, has proven to be the most successful method used in the search for novel pesticides.

Combinatorial chemistry

The novel techniques of combinatorial chemistry were for a period an attractive source of potential leads. The method is based on the generation of a vast but unspecified chemical library, which is then screened. Combinatorial chemistry has found most use in pharmaceutical drug design and its application in the production of peptide libraries is well documented (Nielsen *et al*., 1993). The interest within fungicide discovery lies in the production of arrays of easily synthesized, cheap and relatively low-molecular-weight compounds. Compounds are synthesized on the surface of inert materials or bacteriophages. Of course, there is no guarantee that the compounds produced by this method will be novel; nor does the researcher know the relative amounts of each compound residing on the surface of the support medium. The skill is to be able to combine molecules to establish large libraries that can be screened and then, by a series of elimination studies, the active moieties can be defined and resynthesized in quantity. The advantage of the use of combinatorial chemistry is that huge numbers of chemicals can be screened in specially designed micro-tests at very low cost. Costs rise dramatically only when a particular library is discovered to possess activity.

Analogue synthesis

Analogue synthesis is the practice of synthesizing compounds that retain the important structural core (the pharmacophore) but have different substitutions. Often the identity of the pharmacophore only becomes obvious once several active analogues have been synthesized and tested. Structural features present in active compounds but absent in inactive compounds are likely to be the pharmacophore.

The goal of analogue synthesis is to optimize the activity of compounds defined as leads in the process of screening and is the most successful form of pesticide discovery. It builds on the random screening described above. The leads may be company-owned (in-house) or may be based upon known chemistry ('me-too' synthesis). An example of the inventive scope of me-too fungicide discovery is the development by several companies of the triazole series of fungicides into a family of distinct products (Table 4.6).

All triazoles are designed about a common chemical structure, the 1,2,4-triazole ring, but not all 1,2,4-triazoles are fungicides: paclobutrazole and uniconazole are plant growth regulators and fluchlorazole is a herbicide safener (see Box 4.2 for an explanation of chemical nomenclature rules).

Analogue synthesis would first be carried out by the company that discovered the original lead and would have preceded the first commercialization. Once announced and patented, other companies have the necessary starting information to begin an analogue synthesis programme of their own. As the lead and the pharmacophore would normally be known, this is likely to lead to the synthesis of many active compounds, compared with random synthesis. On the other hand, the potential market will be less because of the market and patent position established by the first company.

Biorational design

All the fungicides available today were discovered by empirical and/or analogue synthesis and there is no doubt that these approaches will continue to be successful. However, the success rate is decreasing. Novel compounds are becoming more difficult to discover by conventional means because of increasingly higher standards of performance, toxicology and environmental safety, and this has encouraged the use of more rational approaches to pesticide discovery. The biorational approach to fungicide discovery demands a complete knowledge of specific metabolic

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Table 4.6. The triazole family of fungicides. (From Oliver and Hewitt, 2014, updated by the authors.)

Compound	Date announced	Company
Triadimefon	1973	Bayer AG
Triadimenol	1978	Bayer AG
Propiconazole	1979	Janssen Pharmaceutica
Bitertanol	1979	Bayer AG
Diclobutrazol	1979	Zeneca Agrochemicals
Flutriafol	1981	Nihon Nohyaku Co. Ltd
Penconazole	1983	Ciba
Azaconazole	1983	Janssen Pharmaceutica
Diniconazole	1983	Sumitomo Chemical Co.
Flusilazole	1984	DuPont
Imibenconazole	1984	Hokko Chemical Industry Co. Ltd
Tebuconazole	1986	Bayer AG
Cyproconazole	1986	Sandoz AG
Myclobutanil	1986	Rohm and Haas Co.
Tetraconazole	1988	Agrimont SpA
Difenconazole	1988	Ciba
Furconazole	1988	Rhône-Poulenc
Epoxiconazole	1990	BASF AG
Hexaconazole	1990	Zeneca Agrochemicals
SSF-109	1990	Shionogi and Co. Ltd
Bromuconazole	1990	Rhône-Poulenc
Fluquinconazole	1992	Schering AG
Metconazole	1992	Shell
Triticonazole	1992	BASF AG
Prothioconazole	2002	Bayer AG
Mefentrifluconazole	2012	BASF AG

processes, including their role in both the pathogen and host, and an ability to use those data in the definition of new target sites. In some cases, computer graphics can be used to construct three-dimensional models of the active sites of target enzymes. The optimum structural requirements of candidate fungicides can be predicted and synthesis resources directed effectively towards the production of potent inhibitors.

Materials synthesized as part of a rational approach to discovery and shown to be active against target enzymes in high-throughput cell-free assays may lack *in vivo* or, more commonly, *in planta* activity. Deficiencies in spectrum – poor transport characteristics and problems of metabolism – have limited the development of rationally designed compounds. The complex barriers to acceptable performance exceed simple biochemical activity and, to date, have prevented the advances made in fundamental molecular design from reaching a commercial end point.

The biorational approach is becoming increasingly significant, optimizing lead chemistry with known MOAs. Its first application was with C14-demethylation inhibitors. Members of this class of fungicides are specific inhibitors of the enzyme P450 14α-demethylase. The three-dimensional structure of the enzyme has been partially solved. Using the known physical and chemical properties of existing inhibitors, the structural requirements for their configuration at the active site of the enzyme have been modelled (Fig. 4.5). This led to the directed synthesis of flutriafol and cyproconazole and the determination of the different binding site of prothioconazole (Parker *et al.*, 2011; Kelly and Kelly, 2013).

Biorational design of the SDHI fungicides

Inhibitors of succinate dehydrogenase have been studied for many decades. The oxathiins carboxin and oxycarboxin were introduced as long ago as 1966 and remain in use because of their low price and continued efficacy against seed-borne bunts and smuts of cereals. However, they have a limited spectrum restricted to seed-borne

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Box 4.2. Nomenclature and classification of fungicides.

Fungicides have a complex vocabulary which acts as a significant barrier to understanding. There are multiple nomenclature systems. These include the FRAC (Fungicide Resistance Action Committee) class, the product name(s), the name of the AI, the formal IUPAC (International Union for Pure and Applied Chemistry) name for the AI, the chemical class (often several levels) and the MOA class. The different names are due in part to different disciplines of people who work in the industry – chemists prefer chemical names, biologists prefer MOA names, farmers and traders prefer product names. To illustrate one example of the confusing possibilities, consider the case of dimethomorph and fenpropimorph. Both are morpholines but the former is an inhibitor of cellulose synthase and acts against oomycetes, whereas the latter is an inhibitor of ergosterol biosynthesis and acts against foliar ascomycetes.

Heterocyclic compounds

Most fungicides are heterocyclic organic compounds. That means they are composed of one (and normally several) cyclic moieties that contain not only carbon but also other elements such as phosphorus, nitrogen and sulfur. They may also be saturated (without double bonds) or unsaturated.

The rules for naming heterocyclic compounds are laid down by IUPAC and follow a series of logical steps. The first level is to count the number of atoms in the ring, the second is whether the ring is saturated, and the third level follows the identity of the hetero atoms. However not all the rules are followed, and exceptions are shown below in italics.

Hetero atom	Prefix
O	Oxa-
N	Aza-
S	Thia-
P	Phospha-

	Fully unsaturated		Fully saturated compounds	
Ring size	With N	Without N	With N	Without N
3	-irine	-irene	-iridine	-irane
4	-ete	-ete	-etidine	-etane
5	-ole	-ole	-otodine	-olane
6	-ine	-in		-ane
7	-epine	-epin		-epane
8	-ocine		-ocin	

Furthermore, some linking letters are omitted to improve pronounceability.

Ring size	Hetero atom	Saturated	FRAC class(es)	Unsaturated	FRAC class(es)
3	N	Aziridine		Azirine	
	N + N	Diaziridine		Diazirine	
	N + O	Oxaziridine			
	O + O	Dioxirane			
4	N	Azetidine		Azete	
	O	Oxetane		Oxete	
	N + N	Diazetidine			
	O + O	Dioxetane		Dioxete	
	S + S	Dithietane		Dithiete	
5	N	*Pyrrolidine*		*Pyrrole* or *azole*	E2
	O	*Tetrahydrofuran*		*Furan*	C2
	S	Tetrahydrothiophene		Thiophene	C7
	S + S	Dithiolane	F2		

Continued

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Box 4.2. Continued.

Ring size	Hetero atom	Saturated	FRAC class(es)	Unsaturated	FRAC class(es)
	N + N	*Imidazolidine* or *pyrazolidine*		*Imidazole* or *pyrazole*	G1
	N + O	Oxazolidine or isoxazolidine		Oxazole or isoxazole	A3
	N + S			Thiazole	I1; P1
	N + N + S			Thiadiazole	P1
	N +N + N			Triazole	G1
	N+N+N+N			Tetrazole	C3; U17
6	N	*Piperidine*	G2	Pyridine	G1
	O	Tetrahydropyran		Pyran	
	N + N	*Piperazine*	G1	Diazine, *pyrimidine* or *pyrazine*	A2; C2; D1; G1
	N + O	*Morpholine*	G2	Oxazine	C3
	O + S			Oxathiin	C2
	N + N + N			Triazine	M8; U35
	N + O + O			Dioxazine	C3

FRAC, Fungicide Resistance Action Committee.

Chemical classifications other than single heterocycles

The chemical classification system for the 200 or more current fungicides focuses on the pharmacophore when that is known. These are often complex heterocyclic structures which are known by trivial names. Other chemical classes refer to small linking groups such as carboxamide. Table 4.7 lists the chemical classifiers and gives examples.

Table 4.7. Major fungicides' irregular pharmacophore classes. (Figures obtained from PubChem.)

Chemical group	MOA class FRAC	Structure	Example active
Anilinopyrimidines	D1		Cyprodinil
Aryl phenyl ketones	B6		Metrafenone

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Box 4.2. Continued.

Chemical group	MOA class FRAC	Structure	Example active
Azanaphthalene	E1		Quinoxyfen
Benzimidazole	B1		Benomyl
Carbamate	F4		Prothiocarb
Cinnamic acid (amide)	H5		Dimethomorph
Dicarboximides	E3		Iprodione

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Box 4.2. Continued.

Chemical group	MOA class FRAC	Structure	Example active
Dithiocarbamate	M03		Mancozeb x:y = 1:0.091
Hydroxy-(2-amino)-pyrimidine	A2		Bupirimate
Hydroxyanilide	G3		Fenhexamid
Phenylamides	A1		Metalaxyl

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Box 4.2. Continued.

Chemical group	MOA class FRAC	Structure	Example active
Phenylpyrroles	E2		Fludioxinil
Quinoline	E1		Quinoxyfen
Spiroketalamine	G2		Spiroxamine

MOA, mode of action; FRAC, Fungicide Resistance Action Committee.

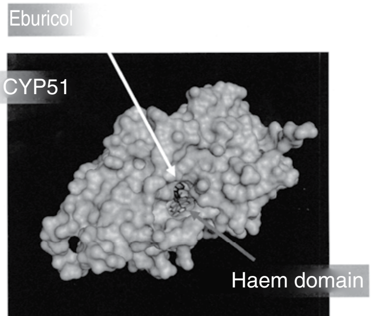

Fig. 4.5. Three-dimensional structure of fungal CYP51 showing the haem active group and the binding site of the substrate eburicol. Such structure allows the *in silico* docking of compounds to predict inhibitory activity prior to the decision whether to synthesize.

basidiomycetes. Attempts to broaden the spectrum resulted in fenfuram, benodanil and mepronil but none of these proved a commercial success. Comparison of these molecules showed that they all shared a structure where two ring moieties were held apart by a rigid planar carboxamide linker, similar to a peptide bond (see Box 4.2). Chemists therefore concentrated on varying the two rings. The breakthrough came with two compounds finally released in 2006. Boscalid used the carboxamide to link a pyridine and a biphenyl group. Bixafen also had a biphenyl group but this was paired with a pyrazole group, a pattern that has been repeated in ten current SDHI products. Pyrazoles are also known as imidazoles, the key pharmacophore of a group of DMI fungicides.

Alterations in the two ring structures have been intensively pursued, resulting in a very successful family of fungicides with broad spectrum and high target selectivity. The enzyme succinate dehydrogenase is composed of four subunits, SDH-A to -D. X-ray crystal structural studies of

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the tetrameric structure were obtained from 2003. They showed that the active site where ubiquinone binds is composed of the B, C and D structures. Inhibitors bind around the active site providing key information for the design of new actives and also rationalizing the evolution of strains with resistance to these inhibitors (Walter, 2010; Xiong *et al.*, 2015).

Biorational design of fenpicoxamid, a new QiI active

The actinomycete bacterial genus *Streptomyces* is an abundant source of natural products, many of which have found to be useful as antibiotics and a few as antifungals. Among these, a product called UK-2A was identified from *Streptomyces* sp. 517-02 in 1996 as a potent inhibitor of fungi (Machida *et al.*, 1999; Ueki *et al.*, 2000). The structure included a picolinamide (pyridine-2-carboxamide) structure and was overall found to be very similar to antimycin A, a classical inhibitor of cytochrome bc1 complex (Fig. 4.6). It was already known that antimycin targeted the Qi site of bc1 and would therefore be unlikely to be affected by mutations that conferred resistance to the strobilurin group of fungicides (C3) that target the Qo site. Picolinamide has been adopted as the chemical class name.

UK-2A was shown to be a very potent inhibitor of SEPTTR and a range of other ascomycete and basidiomycete pathogens, with IC_{50} values between 3 and 20 ppb, but no activity against the oomycetes such as PHTYIN. However, the efficacy in glasshouse trials was much lower as the molecule was readily oxidized and degraded by UV radiation. Meiji Seiki worked with Dow (now Corteva) to derivatize the molecule to improve its stability. UK-2A is currently produced by fermentation of *Streptomyces* sp. 517-02 and readily purified. A single-step modification to add an isopropyl carboxymethyl ether blocking group to the pyridine dramatically increased the stability. The new molecule is called fenpicoxamid (Fig. 4.6) and is being marketed as Inatreq. When incubated in wheat cell cultures or sprayed on plants, fenpicoxamid is taken up and converted by plant oxidases back into UK-2A within 3 h. The efficacy of fenpicoaxmid on infected plants was about 50 times higher than that of UK-2A, whereas it was ten times less potent on SEPTTR *in vivo*. The fungicide has no cross-resistance to QoI or SDHI fungicides and so is expected to have a significant impact. The initial target is SEPTTR on wheat and MYCOFI on bananas. It joins two other molecules that target the Qi site, amisulbrom and cyazofamid. However, unlike these molecules which target oomycetes, fenpicoxamid controls fungi from both the *Ascomycota* and *Basidiomycota* (Owen *et al.*, 2017; Young *et al.*, 2018).

Biorational design of metyltetraprole

Strobilurin fungicides made a sensational impact on the world of fungicides when they were introduced from 1996. The pioneer molecules, azoxystrobin and kresoxim-methyl, possessed a similar structure in which the presumed pharmacophore, a zigzag structure, methoxy-acrylate or oximino-acetate respectively, is linked by an aromatic bridge to a large side chain. Later compounds avoided these structures both to obtain better efficacy but also to avoid patent violations. The molecules were shown to inhibit at the Qo site of the cytochrome bc1 complex (Bartlett *et al.*, 2002). Soon after their introduction, resistance became a major issue. X-ray analysis of binding of mutant versions of the bc1 complex to QoI fungicides showed that while the F129L

Fig. 4.6. Structure of UK-2A, fenpicoxamid and antimycin. (From Owen *et al.*, 2017, with permission.)

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version of the cytochrome b interferes with the binding of the pharmacophore, the G143A version is proximal to the aromatic bridge (Esser *et al.*, 2014). These studies stimulated the testing of new molecules which dispensed with the zigzag pharmacophore altogether (Suemoto *et al.*, 2019). Metyltetraprole is a new QoI fungicide in which the pharmacophore is a pyrrole containing four nitrogen atoms and hence is called a tetrazole. The new molecule is reported to possess excellent activity against ascomycetes but, unlike earlier QoI fungicides, poor activity against basidiomycetes and oomycetes. The efficacy against important, hard-to-control pathogens like SEPTTR and PYRNTE was retained even when used against isolates carrying the G143A and F129L mutations.

Screening methodology

The passage from lead to final product involves a series of screens, one of which is to determine the spectrum of the compound. A testing cascade which forms the screen includes the following activity and performance determinants:

- *activity* – target pathogens and their hosts; and
- *performance* – persistence, application timing and method, mobility and resistance management.

Different target crop–pathogen combinations require particular tests to be carried out to assess the potential value of a candidate fungicide. However, the first steps within the screening process test for activity that can be regarded as essential to further development. Some measure of activity spectrum is implied from the tests. Here the priority is to evaluate the strength of efficacy against target pathogens, compared with the activity of known compounds or standards. At this stage, technical material is used, in a simple formulation such as aqueous acetone, and some weight is given to the fact that this is the lead generation phase of testing: failures to perform to an equivalent level to the standards do not necessarily imply that no further studies should be carried out. However, depending upon the target, high efficacy must be maintained to between 10 and 25 ppm to merit elevation to the next stage of the screen (Fig. 4.7).

The curative properties of compounds are explored early in the selection process. The absence of curative activity is a disadvantage unless some systemicity or the potential to redistribute in the crop is demonstrated. Immobile protectant activity alone limits the use of a candidate to the multi-site-of-action market, dominated currently by cheap and effective materials such as mancozeb. Further development of such compounds is unlikely.

In some crops, especially cereals, it is important that products are effective when applied at volume rates of approximately 250 l/ha. Commonly, screening for cereal fungicides involves a low-volume test that may also present the test compound in an experimental emulsifiable concentrate formulation.

Later tests develop the notion of activity into that of field performance and include formulated material, comparative tests with finished standard products, further spectrum studies and phytotoxicity trials. The failure of a candidate fungicide may result from the absence of a commercially important attribute, such as inadequate mobility, as much as from poor efficacy or phytotoxicity.

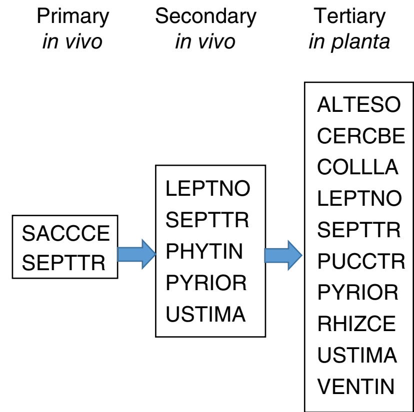

Fig. 4.7. A typical fungicide screening cascade. Initial *in vivo* tests used cells and extracts of SACCCE and SEPTTR. The second stage sought activity against a range of taxonomically diverse but experimentally tractable organisms and included ascomycetes, basidiomycetes and oomycetes. The third level of screen used greenhouse-grown plants of significant targets. Only one obligate pathogen was included to save expenditure and the oomycetes has been ruled out in the secondary screen. (Authors' own data.)

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5

Fungicide Modes of Action and Spectrum

Key Points

- The MOA of a fungicide is defined as the biochemical target of the AI and is the direct cause of the disease control.
- Fungicides can be divided into those with single defined biochemical targets and those that attack multiple sites.
- Single-site fungicides target a small number of basic biochemical functions and often possess a critical chemical group responsible for the activity, the pharmacophore.
- Spectrum is defined as the range of diseases controlled by an AI.
- The spectrum of a fungicide class depends on:
  - the presence of the target site in the pathogen; and
  - whether the physical properties of the AI bring it into contact with the pathogen in infected plants.

Introduction

This chapter describes the biochemical MOAs – *modes of action* – of current fungicides and how that impacts their spectrum. More than 200 chemical compounds have been marketed as fungicides and these compounds can be classified according to either their MOA or their chemical structure. Currently about 130 compounds, belonging to 12 major classes and about 60 chemical groups, are marketed somewhere in the world, and these will be the focus of this chapter.

The classification and naming of fungicides are a source of much confusion. It is important to be aware of the many names used to describe individual and groups of fungicides and to appreciate the significance of the naming conventions, whether they are based on chemical class, biochemical target or agricultural market. Most fungicides are complex organic molecules with several functional groups. Normally just a small part of the molecule binds to the target protein and exerts the inhibitory effect. This structural feature is called the pharmacophore (or sometimes the toxophore). The rest of the molecule functions to hold the pharmacophore in place on the target, direct it to the appropriate cellular compartment and confer adequate stability.

To illustrate the confusion, the pharmacophore for many of the compounds that inhibit the demethylase step in ergosterol biosynthesis is the triazole group and this has been shown to be important in binding to the target enzyme. Thus, the informal name for this group of fungicides is triazoles, a group of molecules that is defined by a five-membered unsaturated ring with three nitrogen and two carbon atoms. None the less, not all triazoles, not even all the ones used

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in agriculture, have this activity. Furthermore, other chemical classes also inhibit the demethylase in a similar manner. In other cases, there is no substantial chemical similarity between actives that have the same MOA; hence it is more common to use the biochemical MOA rather than the chemical structure as the name of the group. This applies to two of the most important classes of fungicides, the QoIs and the SDHIs.

Spectrum is defined as the range of diseases and pathogens that are controlled by an active. One major reason for a limited spectrum is that the target enzyme is present only in some pathogens. For example, oomycetes lack ergosterol but possess cellulose. Fungicides targeting the biosynthesis of these molecules necessarily have a limited spectrum. In addition, the biophysical properties of the entire molecule limit the diseases that are controlled.

Full details of fungicide classes are found in texts such as *Modern Crop Protection Compounds* (Krämer *et al.*, 2012) and *The Pesticide Manual* (Tomlin, 2018). Up-to-date information and the latest decisions on classification are published by the Fungicide Resistance Action Committee (FRAC) whose website (https://www.frac.info, accessed 13 January 2022) is a mine of information. These sources should be consulted for further details. The following is intended as a summary of the most important and interesting classes which are still in widespread use.

Modes of Action

The elucidation of MOAs is a major research preoccupation for the fungicide discovery companies and nowadays the MOA must be determined before the AI can be registered. Any new fungicide lead will be subjected to a battery of tests to determine whether its MOA is different to any of the known ones. If it appears to be new, one method for elucidation is to select for mutants that are resistant and then genetically analyse the basis of the resistance.

Several fungicides have unknown or poorly defined MOAs, but a dozen broad classes and 60 detailed MOAs are described (Table 5.1). The poorly defined groups include the multi-sites, which are believed to simultaneously inhibit several fungal functions.

The broad classes are inhibition of:

A. Nucleic acid synthesis.
B. Cytoskeleton and motor proteins.
C. Respiration.
D. Amino acid and protein synthesis.
E. Signal transduction.
F. Lipid synthesis and membrane integrity.
G. Sterol biosynthesis in membranes.
H. Cell wall biosynthesis.
I. Melanin biosynthesis in the cell wall.
P. Host plant defence activation.
M. Chemicals with multi-site activity.

This list includes many fundamental biochemical functions common to all organisms and shows that the key to success of fungicides is the specificity that enables fungal processes to be inhibited without causing undue damage to the plant hosts and other non-target organisms.

Fungicides are grouped first by target site or, if there are multiple target sites, into one of several multi-site clusters. Most target site groups correspond to a single formal 'group', such as SDHI and QoI; other target site groups are in broad chemical groups, for example the B3 group is divided into benzamides and thiazole carboxamides. Abbreviations for these groups – such as AH, DMI, CAA, QoI, SBI, PA, CAA and SDHI – are widely used in the academic and promotional literature. Many groups are subdivided into a small number of chemical groups. For example, the QoI group is divided into eight chemical groups; DMIs into five. The chemical groups are named according to the common structural element they possess which normally corresponds to the pharmacophore.

A; Inhibition of RNA synthesis

Three fungicide groups target the biosynthesis of RNA. This is a universal biochemical function and so toxicity and specificity must be carefully balanced. The resulting products have rather limited spectrum but provide useful control of oomycetes and powdery mildews.

A1; RNA polymerase 1; phenylamides; PA

These compounds have a phenyl ring connected to an amide nitrogen and include two acylalanines and one oxazolidinone (Fig. 5.1). They are

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Table 5.1. Fungicide classification[a]. (From Oliver and Hewitt, 2014, updated by the authors.)

Mode of action	Code and target site	Group name (abbreviation)	Chemical group	Common name(s)	FRAC code
A; nucleic acid metabolism	A1; RNA polymerase	Phenylamides (PAs)	Acylalanines	Benalaxyl	4
				Metalaxyl	
			Oxazolidinones	Oxadixyl	
	A2; adenosine deaminase	Hydroxy-(2-amino)-pyrimidines	Hydroxy-(2-amino)-pyrimidines	Bupirimate	8
	A3; DNA/RNA synthesis (proposed)	Heteroaromatics	Isoxazoles	Hymexazole	32
B; cytoskeleton	B1; β-tubulin assembly in mitosis	Methyl benzimidazole carbamates (MBCs)	Benzimidazoles	Carbendazim	1
				Thiabendazole	
			Thiophanates	Thiophanate-methyl	1
	B2; β-tubulin assembly in mitosis	*N*-Phenylcarbamates	*N*-Phenylcarbamates	Diethofencarb	10
	B3; β-tubulin assembly in mitosis	Benzamides	Toluamides	Zoxamide	22
	B4; cell division (unknown)	Phenylureas	Phenylureas	Pencycuron	20
	B5; delocalization of spectrin-like proteins	Benzamides	Pyridinylmethyl benzamides	Fluopicolide	43
	B6; actin/myosin/fimbrin function	Aryl phenyl ketones	Benzophenone	Metrafenone	50
			Benzylpyridine	Pyriofenone	
			Aminocyanoacrylates	Phenamacril	47
C; respiration	C2; complex II: succinate dehydrogenase	Succinate dehydrogenase inhibitors (SDHIs)	Phenyl benzamides	Flutolanil	7
				Mepronil	
				Benodanil	
			Phenyl-oxo-ethyl thiophene amide	Isofetamid	
			Pyridinylethyl benzamides	Fluopyram	
			Furan carboxamides	Fenfuram	
			Oxathiin carboxamides	Carboxin	
			Pyrazole-4-carboxamides	Benzovindiflupyr	
				Bixafen	
				Fluxapyroxad	
				Furametpyr	
				Isopyrazam	
				Penflufen	
				Penthiopyrad	
				Sedaxane	
			Pyridine carboxamides	Boscalid	
			N-cyclopropyl-*N*-benzyl-pyrazole carboxamides	Isoflucypram	
			N-methoxy-(phenyl-ethyl)-pyrazole carboxamides	Pydiflumetofen	

Continued

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Table 5.1. Continued.

Mode of action	Code and target site	Group name (abbreviation)	Chemical group	Common name(s)	FRAC code
	C3; complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (*Cytb* gene)	Quinone outside inhibitors (QoIs)	Methoxy acrylates	Azoxystrobin	11
				Coumoxystrobin	
				Enoxastrobin	
				Flufenoxystrobin	
				Picoxystrobin	
				Pyraoxystrobin	
			Methoxy carbamates	Pyraclostrobin	
				Triclopyricarb	
			Methoxy acetamide	Mandestrobin	
			Oximino acetates	Kresoxim-methyl	
				Trifloxystrobin	
			Oximino acetamides	Dimoxystrobin	
			Oxazolidine diones	Famoxadone	
			Dihydrodioxazines	Fluoxastrobin	
		Quinone outside inhibitors (QoI-As)	Tetrazolinones	Metyltetraprole	11A
	C4; complex III: cytochrome bc1 (ubiquinone reductase) at Qi site	Quinone inside inhibitors (QiIs)	Cyanoimidazole	Cyazofamid	21
			Sulfamoyl-triazole	Amisulbrom	
			Picoliamides	Fenpicoxamid	
	C5; uncouplers of oxidative phosphorylation		Dinitrophenyl crotonate	Meptyldinocap	29
			2,6-Dinitroanilines	Fluazinam	
	C7; ATP production (proposed)	Thiophene carboxamides	Thiophene carboxamides	Silthiofam	38
	C8; complex III: cytochrome bc1 (ubiquinone reductase) at Qo stigmatellin-binding subsite	Quinone outside inhibitor, stigmatellin-binding type (QoSI)	Triazolopyrimidylamine	Ametoctradin	45
D; amino acid and protein synthesis	D1; methionine biosynthesis (proposed) (*cgs* gene)	Anilinopyrimidines (APs)	Anilinopyrimidines	Cyprodinil	9
				Mepanipyrim	
				Pyrimethanil	
E; signal transduction	E1; signal transduction (mechanism unknown)	Azanaphthalenes	Aryloxyquinoline	Quinoxyfen	13
			Quinazolinone	Proquinazid	
	E2; MAP/histidine kinase in osmotic signal transduction (os-2, HOG1)	Phenylpyrroles (PPs)	Phenylpyrroles	Fenpiclonil	12
				Fludioxonil	
	E3; MAP/histidine kinase in osmotic signal transduction (os-1, Daf1)	Dicarboximides	Dicarboximides	Iprodione	2
				Procymidone	

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F; lipid synthesis and membrane integrity or function	F3; cell peroxidation (proposed)	Aromatic hydrocarbons (AHs) (chlorophenyls, nitroanilines)	Aromatic hydrocarbons	Quintozene Tolclofos-methyl	14
		Heteroaromatics	1,2,4-Thiadiazoles	Etridiazole	
	F4; cell-membrane permeability, fatty acids (proposed)	Carbamates	Carbamates	Propamocarb	28
	F9; lipid homeostasis and transfer/storage	OSBP1 oxysterol-binding protein homologue inhibition	Piperidinyl thiazole isoxazolines	Oxathiapiprolin Fluoxapiprolin	49
G; sterol biosynthesis in membranes	G1; C14-demethylase in sterol biosynthesis (*erg11*/*cyp51*)	Demethylation inhibitors (DMIs) (steroid biosynthesis inhibitor (SBI) Class I)	Imidazoles	Imazalil Prochloraz Trifluizole	3
			Triazoles	Bitertanol Bromuconazole Cyproconazole Difenoconazole Epoxiconazole Fenbuconazole Fluquinconazole Flusilazole Flutriafol Hexaconazole Ipconazole Mefentrifluconazole Metconazole Myclobutanil Penconaolez Propiconazole Tebuconazole Tetraconazole Triadimefon Triadimenol Triticonazole	
			Triazolinthiones	Prothioconazole	
	G2; Δ^{14}-reductase and Δ^{8}-Δ^{7}-isomerase in sterol biosynthesis (*erg24*, *erg2*)	Amines (‘morpholines’) (SBI Class II)	Morpholines	Dodemorph Fenpropidin	5
			Spiroketalamines	Spiroxamine	

Continued

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Table 5.1. Continued.

Mode of action	Code and target site	Group name (abbreviation)	Chemical group	Common name(s)	FRAC code
	G3; 3-keto reductase, C4-demethylation (*erg27*)	Amines ('morpholines') (SBI Class III)	Hydroxyanilides	Fenhexamid	17
			Aminopyrazolinone	Fenpyrazamine	
H; cell-wall biosynthesis	H5; cellulose synthase	Carboxylic acid amides (CAAs)	Cinnamic acid amides	Dimethomorph	40
				Flumorph	
				Pyrimorph	
			Valinamide carbamates	Benthiavalicarb	
				Iprovalicarb	
				Valifenalate	
			Mandelic acid amides	Mandipropamid	
I; melanin synthesis in cell wall	I1; reductase in melanin biosynthesis	Melanin biosynthesis inhibitors – reductase (MBI-Rs)	Isobenzofuranone	Fthalide	16.1
			Pyrroloquinolinone	Pyroquilon	
			Triazolobenzothiazole	Tricyclazole	
	I2; dehydratase in melanin biosynthesis	Melanin biosynthesis inhibitors – dehydratase (MBI-Ds)	Cyclopropane-carboxamide	Carpropamid	16.2
			Carboxamide	Diclocymet	
			Propionamide	Fenoxanil	
	I3; polyketide synthase in melanin biosynthesis	Melanin biosynthesis inhibitor – polyketide synthase (MBI-P)	Trifluoroethyl carbamate	Tolprocarb	16.3
Unknown	Unknown	Cyanoacetamide-oxime	Benzotriazines	Triazoxide	27
		Benzotriazines	Phenyl acetamide	Cyflufenamid	35
		Phenyl acetamide	Guanidines	Dodine	U06
	Cell membrane disruption (proposed)	Guanidines	Tetrazolyloxime	Picarbutazox	U12
	Unknown	Tetrazolyloxime	Dithiocarbamates and relatives	Mancozeb	U17
				Metiram	
				Propineb	
				Thiram	
				Ziram	
Multi-site	Multi-site contact activity	Dithiocarbamates and relatives	Phthalimides	Captan	M03
				Captafol	
				Folpet	
		Phthalimides	Chloronitriles (phthalonitriles)	Chlorothalonil	M04
		Chloronitriles (phthalonitriles)	Sulfamides	Tolylfluanid	M05
		Sulfamides (electrophiles)	Guanidines	Guazatine	M06
		Guanidines	Quinones (anthraquinones)	Dithianon	M07
		Quinones (anthraquinones)	Quinones (anthraquinones)	Dithianon	M09

FRAC, Fungicide Resistance Action Committee.
[a]Classification of biofungicides and basic substances can be found in Tables 6.1–6.3 (Chapter 6, this volume).

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Metalaxyl-M Benalaxyl-M Oxadixyl

Fig. 5.1. A1 fungicides: metalaxyl-M, benalaxyl-M and oxadixyl. (Based on structures found at PubChem.)

active only against oomycete fungi (Table 5.2) but the basis for the specificity is unknown. They are economically the most important class of fungicides specific for oomycetes. Like many fungicides, metalaxyl and benalaxyl exist as a mixture of enantiomers. It has been established that metalaxyl-M is the more active of the two enantiomers (Nuninger *et al*., 1996). The specific M forms of benalaxyl and metalaxyl are now marketed as kiralaxyl and mefenoxam, respectively.

Phenylamides (PAs) act at specific developmental stages in the oomycete infection process. The release of zoospores from sporangia, their movement, encystment and subsequent germination, as well as the initial penetration and primary haustorium development, are relatively insensitive. However, the development of pathogens beyond the formation of the primary haustorium is well controlled by the PAs. This late but specific inhibition of fungal development is explained by the biochemical MOA. The acylalanines inhibit the synthesis of ribosomal RNA via the RNA polymerase I–template complex (Davidse *et al*., 1988; Randall *et al*., 2014) resulting in the disruption of protein synthesis. In the early life cycle, sporangia and zoospores are sufficiently supplied with ribosomes to permit zoospore formation, germination, penetration and formation of primary haustoria to proceed, even in the presence of PA fungicides. At later stages, continuing inhibition of the RNA polymerase I complex becomes increasingly effective and results in the thickening of hyphal cell walls and eventual cell death. These characteristic symptoms develop through an accumulation of RNA precursors, the nucleoside triphosphates, which promote the activity of fungal β(1,3)-glucan synthetase and the synthesis of cell-wall constituents (Szaniszlo *et al*., 1985).

The PA fungicides are used as protectants and curatives in seed treatments and in root and foliar applications. They are systemic, mainly via the apoplast, but metalaxyl has been reported to move to a limited extent via the symplast. They are active against all oomycete pathogen groups and are suitable for seed, tuber and foliar application. Resistance is a major problem for PA fungicides with cross-resistance between each fungicide.

A2; Adenosine deaminase; hydroxy-(2-amino)-pyrimidines

The hydroxypyrimidines are specific to the control of powdery mildews. They were widely used in the 1970s but have been superseded to a large extent by the DMIs, and by genetic resistance in cereal crops. Only bupirimate (Fig. 5.2) is still in widespread use. It is mainly used to control powdery mildews in apples and ornamentals.

The hydroxypyrimidines inhibit germ-tube elongation and appressorium formation. They act through the inhibition of adenosine deaminase, an enzyme in the purine salvage pathway that recycles the nucleic acid components. Adenosine deaminase is not present in plants but is found in a wide range of fungi. However, it is only the adenosine deaminase activity from powdery mildew fungi that is sensitive to

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Table 5.2. The spectrum[a] of different classes of fungicide. (Authors' own data.)

Mode of action	Group name	OO	B	GFA	GSA	PM	BC	PY
A1	RNA polymerase	A	N	N	N	N	N	N
A2	Adenosine-deaminase	N	N	N	N	A	N	N
A3	DNA/RNA synthesis (proposed)	S	N	N	S	N	N	N
B1	β-Tubulin assembly in mitosis	N	S	A	S	S	S	A
B2	β-Tubulin assembly in mitosis	N	N	N	N	N	A	N
B3	β-Tubulin assembly in mitosis	A	N	N	N	N	N	N
B4	Cell division (unknown)	N	N	N	S	N	N	N
B5	Delocalization of spectrin-like proteins	A	N	N	N	N	N	N
B6	Actin/myosin/fimbrin function	N	N	S	N	A	N	N
C2	Complex II succinate dehydrogenase	N	S	A	S	S	S	S
C3	Complex III cytochrome bc1 QoI	S	S	S	S	S	A	A
C4	Complex III cytochrome bc1 QiI	A	N	S	N	S	S	S
C5	Uncouplers of oxidative phosphorylation	A	N	N	N	S	A	N
C7	ATP transport (proposed)	N	N	N	S	N	N	N
C8	Complex III cytochrome bc1 QoSI	A	N	N	N	N	N	N
D1	Methionine biosynthesis (proposed)	N	N	S	N	S	S	S
E1	Signal transduction (unknown)	N	N	N	N	A	N	N
E2	MAP/histidine kinase os-2	N	S	N	S	S	S	S
E3	MAP/histidine kinase os-1	N	S	N	S	N	S	S
F3	Cell peroxidation	N	S	N	S	N	N	N
F4	Cell-membrane permeability (proposed)	S	N	N	N	N	N	N
F9	Lipid homeostasis (proposed)	A	N	N	N	N	N	N
G1	C14-Demethylase in sterol biosynthesis	N	S	A	S	A	A	A
G2	Δ^{14}-Reductase and Δ^{8}–Δ^{7}-isomerase in sterol biosynthesis	N	S	S	N	A	N	N
G3	3-Keto reductase, C4-demethylation	N	N	N	N	N	A	N
H5	Cellulose synthase	A	N	N	N	N	N	N
I1/2	Melanin biosynthesis inhibitors	N	N	S	N	N	N	A
U06	Unknown phenyl acetamide	S	S	S	S	S	S	S
U12	Guanidines	N	N	N	A	N	N	A
U13	Unknown thiazolidine	N	N	N	N	S	N	N
U27	Cyanoacetamide-oxime	N	N	N	N	A	N	N
U35	Benzotriazine	N	N	N	S	N	N	N
M03	Multi-site dithiocarbamates	A	N	A	A	N	A	N
M04	Multi-site phthalimides	S	N	S	S	N	N	N
M05	Multi-site chloronitriles	N	N	A	N	N	N	N
M06	Multi-site sulfamides	A	N	N	S	N	N	N
M07	Multi-site bis-guazatine	N	N	S	N	N	N	N

[a]A = all, S = some, N = none of the following pathogen subgroups: OO, *Oomycota*; B, *Basidiomycota*; GFA, general foliar *Ascomycota*; GSA, general soil or seed *Ascomycota*; PM, powdery mildew; BC, BOTRCI; PY, PYRIOR.

hydroxypyrimidines, while the enzyme activity from other fungal species is generally not affected.

A3; DNA/RNA synthesis (proposed); heteroaromatics

The isoxazole hymexazole (Fig. 5.2) is the only widely used member of the heteroaromatic class of fungicides in current use. It was commercialized in the 1970s and is sporadically used as a seed and soil/in-furrow fungicide to control damping-off diseases caused by a range of ascomycete fungi and oomycetes such as *Aphanomyces*, *Pythium*, *Fusarium* and *Corticium* in diverse crops such as sugarbeet, rice and tree seedlings. The biochemical MOA is believed to be inhibition of DNA or RNA synthesis but has not been thoroughly investigated.

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Fig. 5.2. A2 and A3 fungicides: bupirimate and hymexazole. (Based on structures found at PubChem.)

B; Mitosis and cell division

B1; β-Tubulin assembly in mitosis; methyl benzimidazole carbamates; MBCs

The MBCs were the first major class of systemic fungicides released and had a revolutionary impact on the industry. They possessed excellent activity against a wide range of ascomycetes and basidiomycetes pathogens but not the rust fungi or oomycetes (Table 5.2) (Delp, 1995). They have protective and eradicant activity against pathogens of cereals, vines, fruit, rice and vegetables and are also used in postharvest treatments. However, resistance has become a major issue in most markets. They are also under suspicion of toxic effects on animals including humans. As a result, MBCs are in decline and used only in niche markets.

The original benzimidazoles were introduced in the 1960s and included benomyl, carbendazim, thiophanate-methyl, fuberidazole and thiabendazole (Fig. 5.3). Benomyl and thiophanate-methyl are pro-fungicides, meaning they are converted in the plant to the AI, in this case carbendazim, another active in this group.

The MOA of the benzimidazoles is well researched and is based on their effects on tubulin integrity. Microtubules are alternating helices of β- and α-tubulins, forming an essential part of the cytoskeleton, and are active in spindle formation and the segregation of chromosomes in cell division. Benzimidazoles disrupt mitosis during cell division at metaphase. The mitotic spindle is distorted, and daughter nuclei fail to separate, resulting in cell death. These morphological changes in treated fungi correlate with biochemical studies that demonstrate the high affinity of benzimidazoles for tubulin proteins in sensitive fungi (Davidse, 1986). Molecular biology techniques confirmed β-tubulin as the target site (Fujimura *et al.*, 1990).

Benzimidazoles are highly selective despite the highly conserved nature of β-tubulins in all eukaryotic organisms. Oomycete fungi and all plants are insensitive to the benzimidazoles. The basis of selectivity probably depends on structural differences at the binding sites of the microtubules. The modification of a single amino acid (from phenylalanine to tyrosine, F200Y; see Box 11.1 in Chapter 11, this volume, for an explanation of this nomenclature) resulting from a mutational change in β-tubulin confers resistance to carbendazim in NEUSCR and many other fungi.

B2; β-Tubulin assembly in mitosis; phenylcarbamates

The phenylcarbamates, as represented by diethofencarb (Fig. 5.3), have a similar action as the MBCs but are active against benzimidazole-resistant fungi (Ishii *et al.*, 1995). This is a rare example of negative cross-resistance (see ‘Cross-resistance’ section in Chapter 11, this volume). Their disruption of mitosis is the same as the

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Carbendazim

Thiabendazole

Thiophanate-methyl

Diethofencarb

Fig. 5.3. B1 and B2 fungicides: carbendazim, thiabendazole, thiophanate-methyl and diethofencarb. (Based on structures found at PubChem.)

benzimidazoles and studies suggest the presence of a common binding region on the β-tubulin protein (Fujimura *et al*., 1990). Diethofencarb is mainly active against BOTRCI, but with useful activity against powdery mildews and is used on grapes and various vegetable crops.

B3; β-Tubulin assembly in mitosis; benzamides

The benzamide fungicide class is currently represented by one product, zoxamide. Like the MBCs, its mode of action is also to bind tubulin (Young *et al*., 2012) (Fig. 5.4). Just one of the zoxamide enantiomers is active and it works by binding the cysteine at position 239 in β-tubulin. Its spectrum is

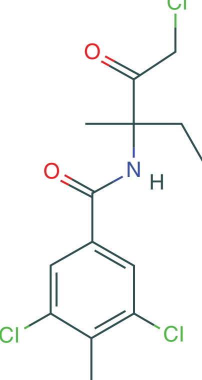

Fig. 5.4. The B3 fungicide zoxamide. (Based on structure found at PubChem.)

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complementary in that it only targets oomycetes. Like the MBCs and phenylcarbamates, resistance is mediated via changes at position 198 and 200 in the oomycete protein. However, resistance has not emerged in the field, possibly due to the diploid nature of oomycete fungi. Zoxamide is a preventive fungicide used to control major oomycete pathogens like PHYTIN, PLASVI and PSPECU.

B4; Cell division (unknown site); phenylureas

The phenylurea pencycuron (Fig. 5.5) is specific for the control of the polyphagous soil pathogen RHIZSO. Its mode of action is via the inhibition of cell division, but details are lacking. We can be sure that it is distinct from those attacking β-tubulin. Resistance has not been detected in the field. It was introduced by BASF in the 1980s and continues to fill a small niche.

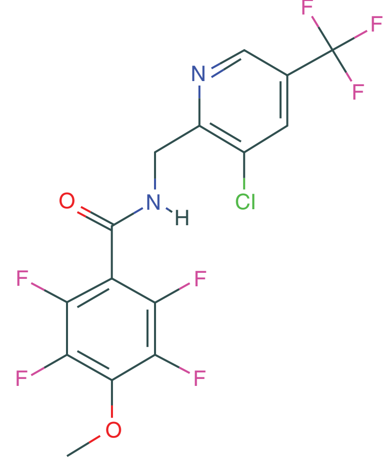
Fig. 5.5. The B4 fungicide pencycuron. (Based on structure found at PubChem.)

B5; Delocalization of spectrin-like proteins; pyridinylmethyl benzamides

Fluopicolide and the not-yet-released fluopimomide (Fig. 5.6) are also benzamides, like B3, but have a distinct MOA. They bind and disrupt the function of spectrin-like cytoskeletal proteins. Spectrins are best studied in human red blood cells. When the cells are washed with detergent, the spectrins form a ghost-like structure, hence the name. Their role is to maintain cellular shape. The pyridinylmethyl benzamides are specific to oomycete diseases and are used as protectant fungicides. They inhibit all stages of growth from germination to mycelial growth and sporulation. Some reports of resistance have emerged.

B6; Actin/myosin/fimbrin function; aryl phenyl ketones

The B6 class of fungicides has little chemical similarity but a common mode of action: disruption of the major cytoskeletal proteins actin, myosin or fimbrin. The aminocyanoacrylate phenamacril (Fig. 5.7) was released in 2014



Fluopicolide

Fluopimomide

Fig. 5.6. B5 fungicides: fluopicolide and fluopimomide. (Based on structures found at PubChem.)

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Phenamacril Metrafenone Pyriofenone

Fig. 5.7. B6 fungicides: phenamacril, metrafenone and pyriofenone. (Based on structures found at PubChem.)

based on its excellent activity against a range of diseases caused by *Fusarium* species. An important feature was that it suppressed the production of mycotoxins during infection. The mode of action was revealed by studying the genetics of resistance. Mutants had altered genes for myosin 5, which plays important roles in cell polarization and cytokinesis. It appears that phenamacril binds to the myosin, preventing the ATPase function that drives the movement of the microfilaments. The specificity is due to the very restricted range of species that possess the methionine at position 375 that confers sensitivity (Zhou *et al*., 2020).

The benzophenone metrafenone and the benzoylpyridine pyriofenone (Fig. 5.7) also have a very limited spectrum, being restricted to powdery mildew diseases. The MOA is believed to be via binding the actin cytoskeleton leading to disruption of hyphal morphogenesis (Opalski *et al*., 2006).

C; Respiration

The mitochondrial respiration chain has proved to be a fertile source of targets. In all eukaryotes, the mitochondrion is the site of energy conversion from sugars to carbon dioxide via the electron transport chain. Electrons flow from NADH and succinate to oxygen to form water, while protons are pumped out across the inner mitochondrial membrane. ATP is formed when protons re-enter via the ATP synthase.

The structure and function of the mitochondrial electron transport chain was largely elucidated by using potent and specific inhibitors to dissect the process of ATP and NAD/PH production from acetyl-CoA. These inhibitors have all been useful leads in the development of many potent and broad-spectrum inhibitors of fungal and oomycete development and many of the steps in the process have been targeted (Fig. 5.8). The electron transport chain is the site of generation of biochemical energy in the form of ATP and so any interruption will inhibit development by restricting the supply of metabolic energy. Furthermore, disruption of the electron transport chain releases highly toxic reactive oxygen species which damage the cell and potentiate the inhibitory effect. Damage to the mitochondrion often leads to the cell undergoing apoptotic cell death.

The mitochondrial respiration chain is ubiquitous in both target and non-target organisms and the protein sequences in the five major complexes (I, NADH dehydrogenase; II, succinate dehydrogenase; III, cytochrome bc1 complex; IV, cytochrome c oxidase; and the proton ATPase) often show high levels of conservation. None the less, specific, highly active and safe inhibitors have been found. Inhibitors of all four electron transport complexes which generate the proton-motive force (PMF), the ATP synthase (which uses the PMF to synthesize ATP), the ATP transporter and uncouplers that collapse the PMF have all developed as fungicides. The major groups of current products inhibit complex II and complex III, with a few products targeting ATP synthase, ATP transport and acting as uncouplers.

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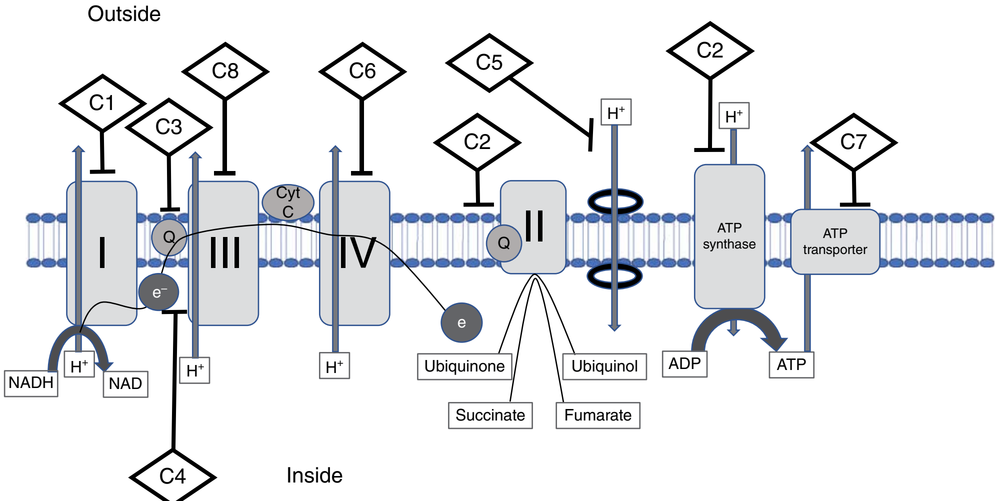

Fig. 5.8. Schematic diagram of the inner mitochondrial membrane. Complexes comprise several proteins plus cytochrome and cofactors. The fungicide target sites are indicated. Complex I, NADH reductase (C1); complex II, succinate dehydrogenase (C2); complex III, cytochrome bc1, QoI (C3), QiI (C4) and QoSI (C8); ATP synthase (C6); ATP transport (C7); and uncouplers which allow protons to re-enter the mitochondrion without passing through the ATP synthase. CytC, cytochrome c; Q, quinone. (Authors' own figure.)

C2; Complex II; succinate dehydrogenase inhibitors; SDHIs

Succinate dehydrogenase occurs in the respiratory chain as part of complex II. The complex contains non-haem iron–sulfur proteins that act in the transfer of electrons from reduced flavin adenine dinucleotide (FAD) to coenzyme Q. Succinate dehydrogenase contributes both to electron transport and the citric acid cycle in that succinate is oxidized to fumarate. As a result, the inhibitors can have potent activity.

The SDHI group of fungicides has a long and interesting history and has recently undergone a major expansion resulting in a wide range of compounds with a broad spectrum and excellent activity. They now rank with QoI and sterol biosynthesis inhibitors in importance and market size.

The first SDHIs were the oxathiin carboxamides, oxycarboxin and carboxin (Fig. 5.9), introduced as long ago as 1966. They were shown to be specific inhibitors of succinate dehydrogenase (Ulrich and Mathre, 1972). The spectrum of the carboxins was limited to seed-borne *Basidiomycota* and they were used mainly as seed treatments to control bunts and smuts diseases of cereals, cotton, oilseed rape and legumes. The limited spectrum and poor mobility meant that they had little overall impact.

A breakthrough came in 2002 with the release of boscalid, a pyridine carboxamide, by BASF (Fig. 5.9). This product has broad-spectrum and foliar activity against a wide range of highly damaging pathogens such as SEPTTR and PUCCRT. The spectrum was thus extended to foliar fungal pathogens, but not to oomycetes. Since then, all the major companies have released SDHIs with complementary activity and mobility characteristics. Major examples include bixafen, isopyrazam, penflufen and pydiflumetofen (Fig. 5.9). Current SDHIs mainly target foliar tissues but others such as sedaxane are use in seed treatments (Fig. 5.9). All the compounds share an amide bond unit surrounded on both sides by aromatic rings of various types and are all believed to bind to the same site in the succinate dehydrogenase complex. This is the ubiquinone-binding pocket (Q site), a hydrophobic region formed at the intersection of the B, C and D subunits. This explains why resistance, which is a significant issue, involves changes in the genes encoding SDH-B, -C and -D.

Modern SDHIs have both preventive and curative action, and most are designed for foliar action. Current recommended SDHIs for wheat in

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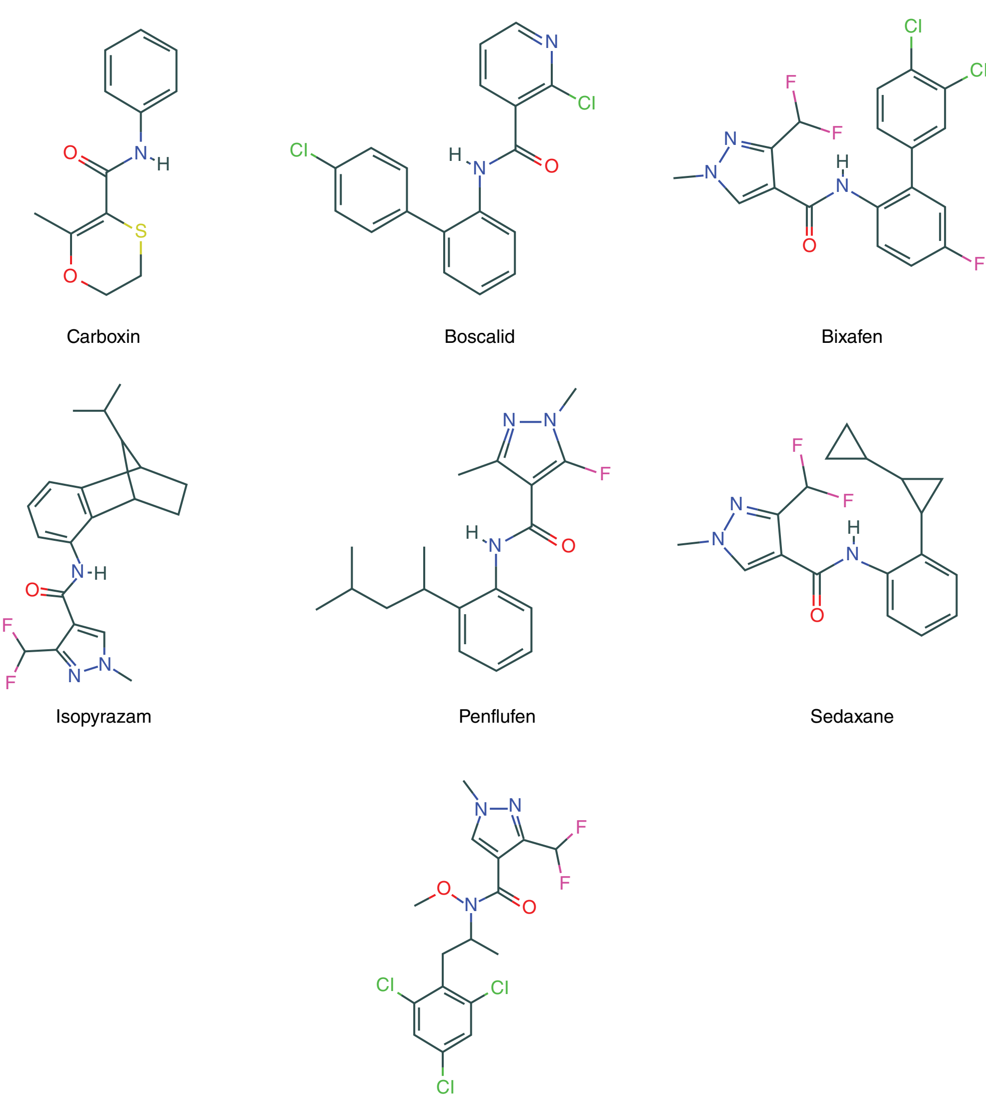

Fig. 5.9. Some SDHI fungicides: carboxin, boscalid, bixafen, isopyrazam, penflufen, sedaxane and pydiflumetofen. (Based on structures found at PubChem.)

Europe include benzovindiflupyr, fluxapyroxad, penthiopyrad, bixafen and isopyrazam. Bixafen is predominantly used on canola, whereas boscalid is used mainly on horticultural crops and turf grass.

C3; Complex III; cytochrome bc1 (ubiquinol oxidase) at Qo site (Cytb *gene); QoI*

This group of fungicides formally called QoIs, but commonly called strobilurins or even 'strobis', was released with great expectations in the 1990s. This class vividly illustrates the highs and lows of the fungicide industry (Bartlett *et al.*, 2002). The compounds have highly potent activity in the parts per billion range and a wide spectrum including oomycete, basidiomycete and all groups of ascomycete fungi. They are exceptionally non-toxic to non-target organisms including most plants and they are rapidly degraded in soil, making them environmentally

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benign. Their Achilles' heel has been resistance which rapidly became a major issue. Despite this, the class includes several fungicides with annual sales approaching $1 billion and remains one of the three most important classes of fungicides.

The sequence of events which led to the development of the strobilurins as agricultural fungicides began in the 1960s, with the discovery by a Czech scientist, Vladimir Musilek, of a naturally occurring enol-ether stilbene called strobilurin in the wood-rotting basidiomycete fungus *Strobilurus tenacellus*. This was developed for use as a medicinal agent to treat skin diseases. The *in vivo* antifungal activity of strobilurin A was published in 1977 (Anke *et al.*, 1977) (Fig. 5.10) and the MOA was shown to be the inhibition of electron transfer in complex III of mitochondrial respiration (Becker *et al.*, 1981). In 1983, BASF began to examine the potential of the strobilurins as precursors for new synthetic pesticides. Although strobilurin A had good *in vivo* activity and an unusually broad spectrum, it possessed only weak activity *in planta*. It was hypothesized that the poor transference of activity from *in vivo* to *in planta* tests was due to the instability of the molecule, permitting rapid degradation through photolysis or metabolism. A synthesis programme was initiated to increase stability and thereby optimize *in planta* activity. At much the same time, ICI Plant Protection (now Syngenta) investigated the activity of oudemansin A, an oxime ether discovered in another basidiomycete fungus, *Oudemansiella mucida* (Beautement and Clough, 1987; Beautement *et al.*, 1991). This work also led to the production of a series of analogues to improve stability (Fig. 5.10). The progression to a final product became a race and both companies filed patents separated by just 2 days (Sauter *et al.*, 1999).

The preferred compounds arising from the modification of the patented oxime ethers were kresoxim-methyl and azoxystrobin (Ammermann *et al.*, 1992; Godwin *et al.*, 1992) (Fig. 5.10). Both proved to be highly active compounds with broad use in a very wide range of crops and diseases. Azoxystrobin is effective against pathogens from all groups but particularly in the control of downy and powdery mildews of grapevine. In contrast, kresoxim-methyl is more effective than azoxystrobin against cereal powdery mildew. Although they work best as preventives, they have eradicant activity against most target diseases.

It is remarkable that these compounds have activity against pathogens from the *Ascomycota*,

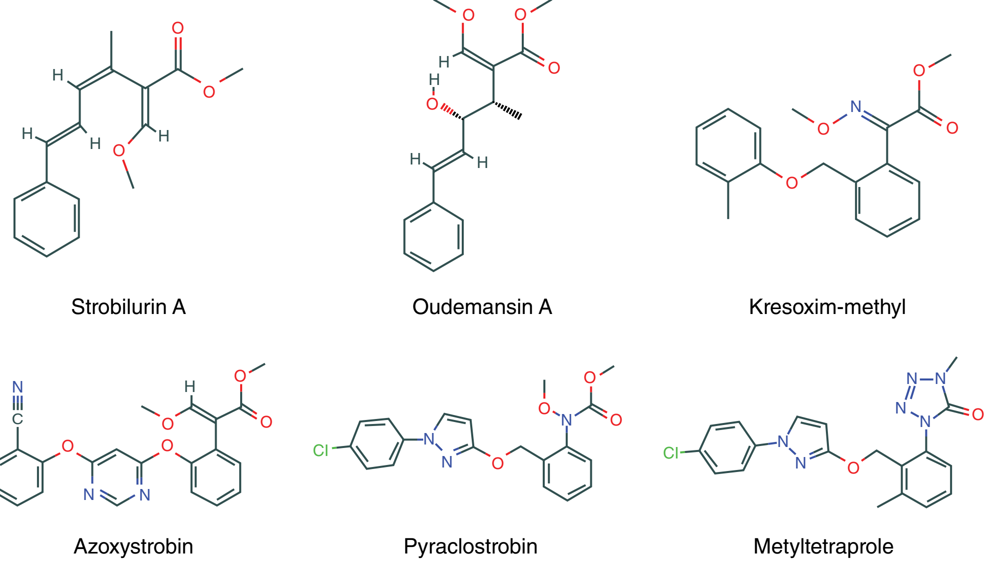

Fig. 5.10. Some C3 QoI fungicides: strobilurin A, oudemansin A, kresoxim-methyl, azoxystrobin, pyraclostrobin and metyltetraprole. (Based on structures found at PubChem.)

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Basidiomycota and *Oomycota* but are very safe for both plants and animals. All the main companies have developed analogues with the same MOA, albeit the chemical structures differ notably. The market leaders now are azoxystrobin, pyraclostrobin and trifloxystrobin, although about ten compounds are currently marketed all over the world.

QoIs inhibit electron transfer in complex III (the bc1 complex) of the mitochondrial electron transport chain (Fig. 5.8). Spore germination is the developmental stage with most sensitivity to QoIs because mitochondrial oxidative phosphorylation of internal spore storage compounds such as lipids, sugar alcohols and glycogen fuels early development before pathogens acquire nutrients from their host or environment. The compounds possess slow-acting systemic properties and can provide long-term disease control. Redistribution within the crop is achieved through a continuous mechanism of absorption from the waxy cuticular layer of leaves into the plant and through movement via the vapour phase and reabsorption into cuticular waxes. It was also noted that treated plants stayed green for longer after spraying, leading to significantly higher yields even in the absence of disease. The exact mechanism of this effect is still under discussion, but it is large enough to pay for the cost of application in high-yielding situations.

Resistance to strobilurins was revealed less than 2 years after release. Cereal powdery mildew isolates with very high resistance were observed and these had a consistent pattern of mutation in the cytochrome b (*Cytb*) gene encoding one of the subunits of complex III. All extant QoIs were cross-resistant, necessitating the introduction of resistance management practices (see 'Cross-resistance' section in Chapter 11, this volume).

C4; Complex III; cytochrome bc1 (ubiquinone reductase) at Qi site; QiI

Three current compounds target another haem centre in complex III known as the Qi (inside) site. The spectrum of cyazofamid and amisulbrom is limited to oomycetes and they are used to control PHYTIN and PLASVI, whereas the recently introduced fenpicoxamid is active against ascomycete fungi and especially SEPTTR (Mitani *et al.*, 2001; Owen *et al.*, 2017; Fontaine *et al.*, 2019) (Fig. 5.11). They are susceptible to resistance but are active against QoI-resistant isolates. They are structurally unrelated and applied as foliar sprays with mainly preventive activity.

C5; Uncouplers of oxidative phosphorylation

The role of the electron transport chain is to generate the electromotive force, via displacement of protons, which will drive the synthesis of ATP. Uncouplers are compounds that interfere with ATP synthesis by collapsing the electromotive force. They do this by inserting into the inner mitochondrial membrane and providing a pathway for the transport of protons down the concentration gradient. The classic compound used to prove the chemiosmotic theory was dinitrophenol. In view of this rather non-specific MOA, it is not surprising that most uncouplers are too toxic for current use. Fluazinam, a diarylamine (Fig. 5.12), has low mammalian toxicity because it is metabolized by animal tissues into innocuous products. The compound, released in 1990, has become commercially very significant as a protectant fungicide used in the control of BOTRCI, *Sclerotinia*, *Alternaria*, *Colletotrichum*, PHYTIN and VENTIN. It also controls brassica clubroot caused by the non-fungus PLADBR. It is not systemic but can be used both as a foliar spray and for seed treatments. The parent compounds are unstable to chemical hydrolysis and, following uptake into fungi, undergo enzymatic hydrolysis to yield the toxic dinitrophenols, which then act as uncouplers or inhibitors of mitochondrial oxidative phosphorylation.

Meptyldinocap (Fig. 5.12) is a dinitrophenol crotonate that was released in 2007 and was used as a protectant, curative and eradicant fungicide for controlling powdery mildew on grapevines. It was derived as a single isomer of the discontinued AI dinocap but with adequately low animal toxicity.

C7; ATP transport (proposed); thiophene carboxamides

Silthiofam is the sole example of a fungicide that interferes with the process of ATP transport (Fig. 5.12). Most ATP in a pathogen cell is synthesized on the inside of the mitochondria using the PMF. To get to the cytoplasm, it must be exported. Silthiofam, a thiophene carboxamide,

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Cyazofamid

Amisulbrom

Fenpicoxamid

Ametoctradin

Fig. 5.11. More complex III fungicides: C4 QiIs, cyazofamid, amisulbrom and fenpicoxamid; C8 QoI, ametoctradin. (Based on structures found at PubChem.)

is specific for the control of the cereal root disease take-all. It is applied to the seed.

C8; Inhibition of complex III; cytochrome bc1 (ubiquinone reductase) at Qo stigmatellin-binding subsite; QoSI

Ametoctradin is another new fungicide targeting complex III. Assays suggest that this triazolopyrimidine can bind to either of the Qo and Qi sites depending on the redox status of the mitochondrial chain. Its binding most resembles the inhibitor stigmatellin; hence the description of the MOA as QoSI (Fig. 5.11). Its spectrum is limited to oomycetes, and it is used to control PHYTIN and PLASVI on potatoes, grapes and many vegetables (Zhu *et al.*, 2015; Dreinert *et al.*, 2018).

D; Amino acid and protein synthesis

D1; Methionine biosynthesis (proposed) (cgs gene); anilinopyrimidines; APs

The anilinopyrimidines (APs) mepanipyrim, pyrimethanil and cyprodinil, also known as the pyridinamines (Fig. 5.13), are an ascomycete-specific group and have extensive use in a wide variety of crops since the 1990s. Mepanipyrim and pyrimethanil are active against BOTRCI and VENTIN. Cyprodinil has additional activity against foliar ascomycetes including powdery mildews especially for use on cereals. They are mostly used nowadays on fruit and vegetables.

The MOA was linked to methionine biosynthesis inhibition because inhibition could be

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Fluazinam Meptyldinocap Silthiofam

Fig. 5.12. C5 and C7 fungicides: the uncouplers fluazinam and meptyldinocap; and silthiofam, an inhibitor of ATP transport (proposed). (Based on structures found at PubChem.)

Mepanipyrim Pyrimethanil Cyprodinil

Fig. 5.13. D1 fungicides: the anilinopyrimidines mepanipyrim, pyrimethanil and cyprodinil. (Based on structures found at PubChem.)

relieved by adding sulfur-containing compounds like cystathionine. Furthermore, resistance was associated with alterations in the promoter of the cystathionine-γ-synthase gene. However, a recent study shows that APs disrupt a range of mitochondrial functions and that resistance is linked to several genes all linked in various ways to mitochondrial activities (Mosbach *et al.*, 2017).

E; Signal transduction

E1; Signal transduction (mechanism unknown); azanaphthalenes

Quinoxyfen and proquinazid (Fig. 5.14) are specific powdery mildewcides. They are azanaphthalenes but otherwise have little structural similarity. It is likely that they have a common target site as cross-resistance has been found. Quinoxyfen was announced by DowElanco in 1996 (Hollomon *et al.*, 1997) and is unusual in its action as a systemic protectant which provides long-term control of cereal mildew. The movement of quinoxyfen through leaf sheaths to the developing basal meristem and hence to leaves not directly exposed to treatment may be involved, and other redistribution via the vapour phase may also provide a route for compound redistribution in crops. Proquinazid was introduced in 2005 by DuPont. Quinoxyfen inhibits appressorium formation by disrupting signal transduction processes (Lee *et al.*, 2008).

E2; MAP/histidine kinase in osmotic signal transduction (os-2, HOG1); phenylpyrroles; PPs

Pyrrolnitrin is a secondary metabolite formed by *Pseudomonas pyrrocina* that has antifungal

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properties but is unsuitable for use in practical disease control because of its instability in light. Optimization of pyrrolnitrin led to the discovery of the commercial fungicide fludioxonil (Fig. 5.14). The MOA appears to involve the MAP (mitogen-activated protein) kinase HOG1 (also known as os-2) (Irmler *et al.*, 2006). HOG1 is a yeast gene involved in the response of cells to osmotic stress, but HOG1 orthologues in other fungal species are involved in the response to numerous environmental signals.

The phenylpyrroles (PPs) have a broad fungal disease control spectrum but are inactive against oomycete fungi. Fludioxinil is used mostly on fruit and vegetables for diseases such as of seedlings, stem bases and in storage.

E3; MAP/histidine kinase in osmotic signal transduction (os-1, Daf1); dicarboximides

Dichlozoline was the earliest commercial dicarboximide and was used in the control of *Sclerotinia* and BOTRCI. Current compounds include iprodione and procymidone (Fig. 5.15). The dicarboximides inhibit spore germination and cause hyphal branching, swelling and lysis. Like PPs, the MOA involves interference with MAP kinase signalling, in this case the osmosensing histidine kinase known as os-1 or Daf1 (Oshima *et al.*, 2002).

The spectrum of the group includes BOTRCI, SEPTTR and other foliar ascomycetes in cereals, grapevine, canola, hops, ornamentals, fruit, legumes and vegetables. Iprodione is also used as a

Quinoxyfen Proquinazid Fludioxinil

Fig. 5.14. Signal transduction inhibitor fungicides. E1, quinoxyfen and proquinazid; E2, fludioxinil. (Based on structures found at PubChem.)

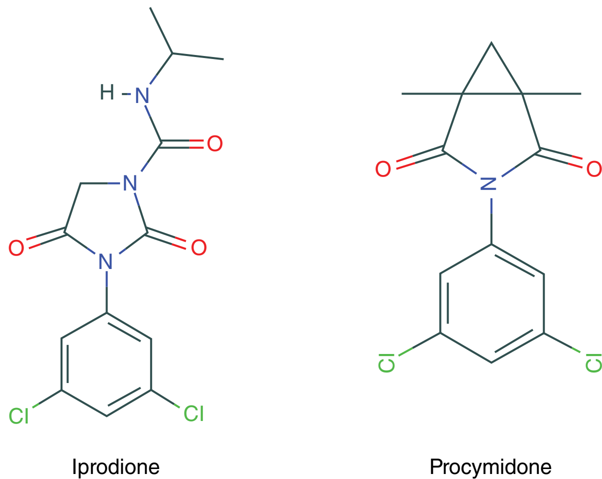

Fig. 5.15. E3 dicarboximides: iprodione and procymidone. (Based on structures found at PubChem.)

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postharvest fungicide for fruit and vegetables. Procymidone is additionally used as a seed dressing.

F; Lipid synthesis or transport/membrane integrity or function

F3; Cell peroxidation (proposed); aromatic hydrocarbon (AH) fungicides (chlorophenyls, nitroanilines), 1,2,4-thiadiazoles

The MOA of three fungicides discovered in the 1950s and 1970s, quintozene, etridiazole and tolclofos-methyl, has not be fully characterized, but is believed to be peroxidation of cell-membrane lipids. Quintozene and tolclofos-methyl are simple chlorophenols and etridiazole is a thiadiazol (Fig. 5.16). Despite their small size and generalist MOA, they remain in use as contact fungicides. Etridiazole is used to control oomycetes while quintozene and tolclofos-methyl are used for ascomycete damping-off diseases.

F4; Cell-membrane permeability, fatty acids (proposed); carbamates

Propamocarb is another long-established fungicide with a poorly defined MOA (Fig. 5.17). It is believed to induce cell membrane permeability. It is still in use to control oomycetes on tobacco, potatoes, turf and ornamentals.

F9; Lipid homeostasis and transfer/storage; OSBP1 oxysterol-binding protein homologue; piperidinyl thiazole isoxazolines

Two new fungicides to control oomycetes, oxathiapiprolin and fluoxapiprolin, have recently been introduced by DuPont (now Corteva) and Bayer, respectively, with an interesting and new MOA (Fig. 5.17). To determine the MOA, molecular tags were added to the chemical and these were found to bind specifically to a protein thought to bind oxysterol. Mutants in PHYTCP that were resistant were found to have a single alteration in a gene encoding an oxysterol-binding protein (OSBP). When the mutated version was expressed in wild-type cells, the transformants were found to be resistant. This is a new target for oomycete control, and it is not yet clear what the main role of the OSBP1 protein is and why the fungicide is so active against *Phytophthora* and downy mildew pathogens (Pasteris *et al.*, 2016). It is not active against *Pythium* species.

These piperidinyl thiazole isoxazolines control a wide range of oomycete diseases. They have both preventive and curative activity. Both mycelial growth and sporangia formation are inhibited.

G; Sterol biosynthesis in membranes (SBIs)

Sterols are core components of cell membranes along with phospholipids and are present in all eukaryotes. Cholesterol is the main sterol in animals, whereas in fungi sterol biosynthesis is carried out *de novo* from acetyl-CoA to produce the principal sterol in most fungi, ergosterol (Fig. 5.18). Chemicals that inhibit sterol biosynthesis are very effective crop disease control agents. They constitute the single largest group of fungicides in terms of the number of current individual actives and are one of the top three classes of

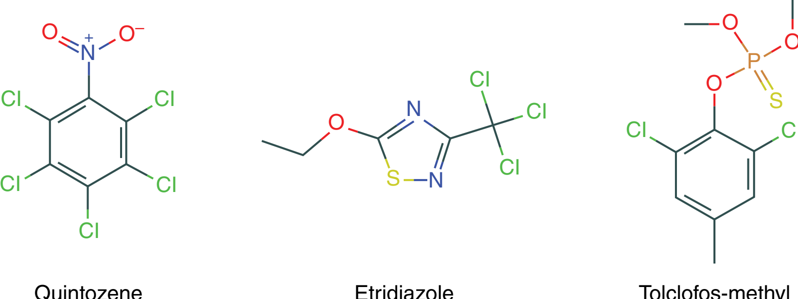

Fig. 5.16. F3 fungicides: quintozene, etridiazole and tolclofos-methyl. (Based on structures found at PubChem.)

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Propamocarb

Oxathiapiprolin

Fluoxapiprolin

Fig. 5.17. F4 and F9 fungicides: propamocarb and the oxysterol-binding protein homologues oxathiapiprolin and fluoxapiprolin. (Based on structures found at PubChem.)

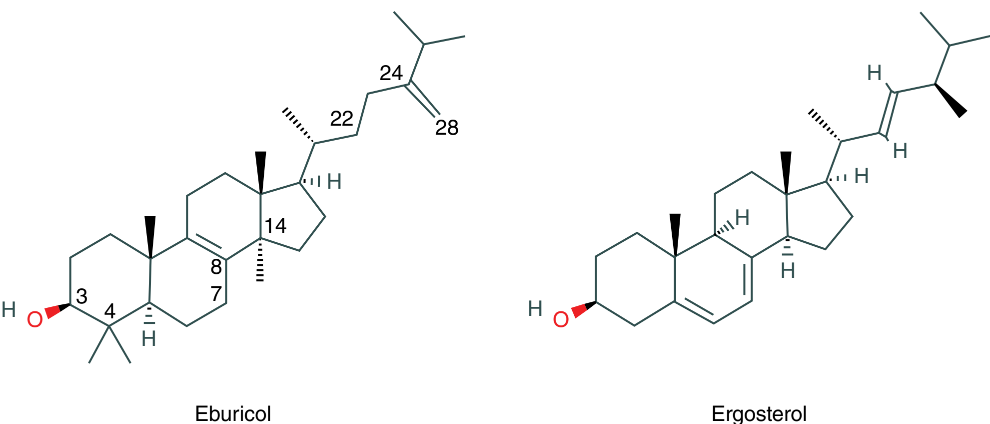

Fig. 5.18. Eburicol and ergosterol. The numbered carbon atoms are modified by enzymes targeted by fungicides. (Based on structures found at PubChem.)

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fungicide in terms of sales. Steroid biosynthesis inhibitors (SBIs) are systemic and provide protectant, curative and eradicant control. They do not inhibit spore germination, because spores typically contain adequate stores of sterols that can supply the growing mycelium for a day or two.

The biosynthetic pathway to ergosterol and the sterol variants is a feature of all true fungi but is absent from the *Oomycota*, which cannot synthesize sterols but instead obtain them directly from their hosts through mycelial uptake. This difference is the basis of the selectivity of SBIs, which cannot be used for the control of oomycete diseases. The sterol composition of fungal pathogens is complex. In most cases there is one major sterol comprising about 50% but with several other slightly different compounds making up the rest. Ergosterol is the main sterol in most ascomycetes, but powdery mildews have 24-methylene-cholesterol. Among basidiomycetes the smuts have ergosterol, but rusts have stigmasta-7,24(28)-dienol (Weete *et al.*, 2010). These fungal sterols play a critical role in the maintenance of membrane function: a reduction in ergosterol availability results in membrane disruption and electrolyte leakage.

The biosynthetic route is not necessarily linear, and it is more accurate to think of the various enzymes as 'decorating' the sterol backbone as and when a suitable substrate is available. The order of these decorations is not fixed so that a very large number of compounds can be detected particularly when individual steps are partially inhibited. A reduction in sterol biosynthesis leads to membrane leakage due to lack of the final product. In addition, inhibition of the pathway leads to the accumulation of shunt compounds which are often significantly toxic, adding to the antifungal effect.

Inhibitors of sterol biosynthesis were discovered and developed to combat human fungal diseases, but similar compounds quickly became available in crop protection and their introduction in the late 1960s heralded a radical change in the management of crop disease. The pathway for ergosterol biosynthesis has been established best in the yeast SACCCE. Yeast *ERG* genes control the biosynthesis and have homologues in other species (Table 5.3). The details of the biosynthetic pathways differ slightly in other fungi. Fungicides that act through the inhibition of the sterol pathway can be divided into four major classes (G1–G4 and SBI Class I–IV) and further subdivided by which enzyme is inhibited.

*G1; C14-demethylation inhibitors (*erg11/cyp51*); SBI Class I; DMIs*

The most important SBIs are the C14-demethylation inhibitors (DMIs), group G1, sometimes called the triazoles, although this is only one of the chemical classes in this group. The commercial strength of the DMIs arises from their activity spectrum and utility, which is very wide, with uses against most major ascomycete and basidiomycete pathogens (Table 5.2) but not oomycetes. The DMIs inhibit the removal of the C14-methyl group from either lanosterol or eburicol (Fig. 5.18).

Table 5.3. Enzymes and corresponding genes catalysing steps in the generic fungal ergosterol biosynthesis pathway that are targeted by fungicides. (Based on Debieu and Leroux, 2015.)

Enzyme	Gene designation	FRAC class
Squalene monooxygenase	*ERG1*	G4; SBI-IV
Lanosterol synthase	*ERG7*	–
Sterol C24-methyl transferase	*ERG6*	–
Sterol C14-demethylase	*ERG11*, *Cyp51*	G1; SBI-I
Sterol C14α-reductase	*ERG24*	G2; SBI-II
Sterol C4-methyloxidase	*ERG25*	–
Sterol C3-dehydrogenase	*ERG26*	–
Sterol C3-keto reductase	*ERG27*	G3; SBI-III
Sterol $\Delta^8 \rightarrow \Delta^7$-isomerase	*ERG2*	G2; SBI-II
Sterol C5-desaturase	*ERG3*	–
Sterol C22-desaturase	*ERG5*	–
Sterol $\Delta^{24(28)}$-reductase	*ERG4*	–

FRAC, Fungicide Resistance Action Committee.

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The subsequent accumulation of precursor sterols and reduction in ergosterol is thought to be the basis of DMI activity.

There are a few problems. Resistance has developed albeit it took 30 years before serious field failures were noted. Some of the triazoles have poor environmental toxicity profiles and are targeted for removal in the European Union (EU). Phytotoxicity in the form of plant growth regulator effect can be a problem, limiting their use on legume crops; care must be taken when using on ornamental crops.

The target site of the DMIs is the CYP51 enzyme, a b-cytochrome P450 monooxygenase containing a haem group at the active centre. The fungicides appear to bind at the active site, thereby directly inhibiting access of the substrate to the enzyme (Kelly and Kelly, 2013). This both reduces ergosterol synthesis and leads to the accumulation of toxic intermediates (Joseph-Horne *et al.*, 1996). The nitrogen-containing heterocycle binds to the haem, complexing the iron atom at the centre.

Different species of fungi have one, two or even three paralogues (copies of genes that arose from gene duplication) of the *Cyp51* gene – *Cyp51A*, *B* or *C* – and sometimes multiple copies of individual paralogues (Fan *et al.*, 2013; Mair *et al.*, 2019). The presence of the different paralogues accounts for some of the variation in sensitivity in different species to different DMIs. Resistance has become a significant issue and is associated with changes in the coding sequences and overexpression of genes.

Several classes of chemical have been marketed as DMIs but only two are still in widespread use (Table 5.1): triazoles and imidazoles. This diverse range of chemistry is characterized by a nitrogen-containing heterocycle attached to lipophilic side groups. The imidazoles prochloraz and imazilil (Fig. 5.19) are mainly used for foliar and seed applications, respectively. The development of resistance differs from the triazoles, meaning they can be useful mixing partners.

The triazole group contains more than 25 chemicals and nearly all are still in use. It includes ones recommended for seed treatment (e.g. triadimenol and fluquinconazole) and for foliar treatment. New triazoles have been added to the market in waves since the 1970s from the different companies. The first wave was dominated by triadimenol and triadimefon. The second wave (1980s) was led by propiconazole and cyproconazole. Next came epoxiconazole in 1994, still the market leader in some markets but being withdrawn in Europe. Bayer released prothioconazole in 2004. The supplied product, a 1,2,4-triazole-3-thione, is an example of a pro-fungicide. The compound is activated by exposure to the plant, losing the thio group in the process and forming a triazole. The most recent entrant is mefentrifluconazole introduced in 2019 by BASF with a better safety profile than epoxiconazole (Ishii *et al.*, 2021) (Fig. 5.19).

The DMIs have excellent activity in both foliar and seed applications. They are still the mainstay of the control of the major cereal diseases such as SEPTTR, PUCCRT, RHYNSE and PYRNTE, as well as in grapevines for BOTRCI and UNCINE and in bananas for MYCOFI. Resistance is an issue especially in North-West Europe and New Zealand and on banana plantations, but they are performing robustly outside these areas. Being such an old class, many actives have been off-patent for decades and are therefore available in many generic formulations providing cost-effective options to farmers.

*G2; $\Delta^8 \rightarrow \Delta^7$-isomerase and Δ^{14}-reductase inhibitors (*erg24*,* erg2*); SBI Class II; amines (morpholines)*

The G2 class of SBIs (also known as SBI Class II or morpholines or amines) inhibits at least two steps in the biosynthesis of ergosterol, the $\Delta^8 \rightarrow \Delta^7$-isomerase and Δ^{14}-reductase. First developed as long ago as 1964, currently three compounds are in widespread use. These are the morpholine dodemorph, the piperidine fenpropidin and the spiroketalamine spiroxamine (Fig. 5.20). Although the inhibition of the isomerase and reductase has been demonstrated in laboratory studies (Baloch and Mercer, 1987; Steel *et al.*, 1989; Ziogas *et al.*, 1991; Debieu *et al.*, 1992), the comparative importance of the two targeted steps is not well understood and the implications of inhibition are not clear. In addition, although some studies have been carried out that demonstrate the disruptive effects of fenpropimorph treatment on sterol levels and membrane integrity in yeast, *Saccharomyces cerevisiae* (Steel *et al.*, 1989), other work showed that survival was independent of $\Delta^8 \rightarrow \Delta^7$-isomerase activity (Ashman *et al.*, 1991). Morpholine inhibition of

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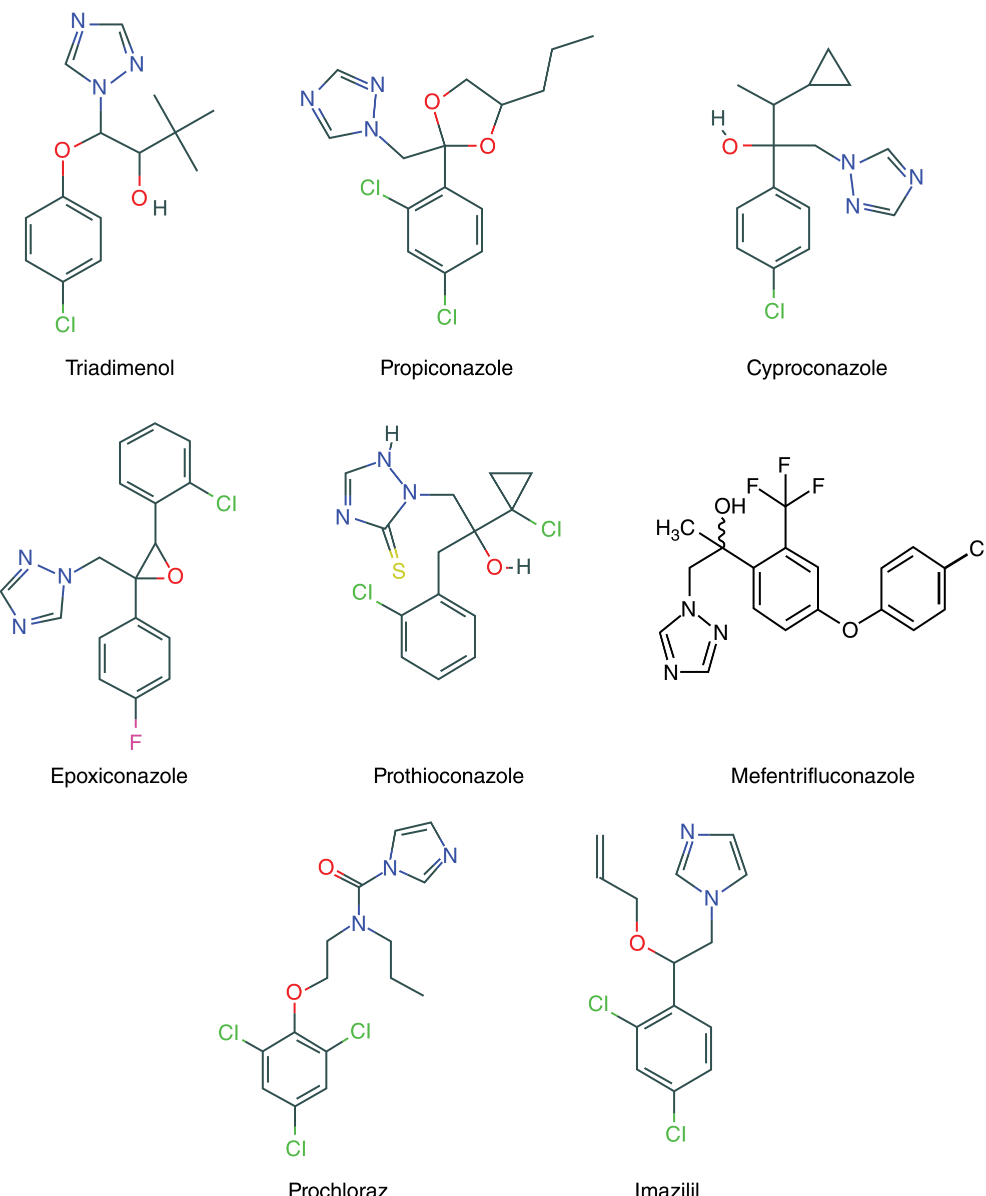

Fig. 5.19. Important DMI fungicides: triadimenol, propiconazole and cyproconazole, epoxiconazole, prothioconazole and mefentrifluconazole; and the imidazoles prochloraz and imazilil. (Based on structures found at PubChem.)

$\Delta^{24(28)}$-reductase, Δ^{24}-transmethylation and squalene-cyclization steps have also been cited as possible MOAs (Debieu *et al.*, 2000). The MOA is mediated by the interaction of the negatively charged enzyme site and the positively charged nitrogen atom in the fungicide molecule. Optimization of activity through structural modification extends to the choice of stereoisomer. In the spiroketal, spiroxamine, the two *cis* forms are more active than the two *trans* isomers (Krämer *et al.*, 1999).

The spectrum is limited compared with the C14-demethylation inhibitors, their major use being against the powdery mildews (Table 5.2).

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Dodemorph

Fenpropidin

Spiroxamine

Fig. 5.20. G2 $\Delta^8 \rightarrow \Delta^7$-isomerase and Δ^{14}-reductase inhibitors: dodemorph, fenpropidin and spiroxamine. (Based on structures found at PubChem.)

Spiroxamine, the newest member of the group (1997), has preventive, curative and eradicant activity against mildew as well as significant activity against other fungi such as rusts and leaf blotches.

*G3; 3-Keto reductase (*erg27*); KRI (keto reductase inhibitor) fungicides; SBI Class III*

The G3 class of fungicides inhibit the 3-keto reductase step in sterol biosynthesis encoded in yeast by *ERG27*. In a typically serendipitous manner, compounds being synthesized by Bayer as herbicides were found to have activity against BOTRCI. Optimization led to the release of the hydroxyanilide, fenhexamid, in 1998 (Fig. 5.21). It was subsequently shown that the compound inhibited a novel site in the sterol biosynthetic pathway, the keto reductase activity on the C3 ketone group (Debieu *et al.*, 2001). Fenhexamid has good activity against BOTRCI and the close relative *Sclerotinia* but only weak activity against other ascomycetes. It is used as a foliar product and has curative activity only. The compound is not translocated so it is used solely as a protectant. Usage rates are high at up to 1 kg/ha. Fenpyrazamine, an aminopyrazolinone (Fig. 5.21), has recently been added to this group with similar spectrum but is claimed to be able to translocate across the leaf giving it curative activity.

Spore germination is not affected but mycelial elongation is inhibited, and this is thought to be the biological MOA. The manner of the inhibition of the keto reductase has not yet been elucidated. Both are used in grapevines and other horticultural crops to control BOTRCI (Debieu *et al.*, 2013).

H5; Cell-wall biosynthesis; carboxylic acid amides; CAAs

The main cell-wall component of oomycetes is cellulose, like plants and unlike fungi which base their cell walls on chitin. Cellulose synthase has proved to be the target site of a diverse group of fungicides with specific activity against oomycetes. They were combined into a coherent group by FRAC in 2005 and called the carboxylic acid amides (CAAs). As cellulose is absent from true fungi, this explained the limited spectrum. The application for CAAs is dominated by PHYTIN and PLASVI and the group has the second largest market size after the PAs.

The group includes one cinnamic acid amide (dimethomorph), three valinamides (iprovalicarb, benthiavalicarb and valifenalate) and a mandelic acid amide (mandipropamid) (Fig. 5.22). The rationalization of this group and the determination of the MOA emerged from studies of resistance as compounds from three chemical classes showed cross-resistance. Note that the CAA abbreviation is meant for carboxylic not cinnamic acid amide. The MOA was fully characterized by identifying mutations in the *CesA3* gene of PHYTIN that conferred resistance to the group (Blum *et al.*, 2012; Gisi *et al.*, 2012).

The CAA fungicides have preventive and some eradicant activity due to some translaminar

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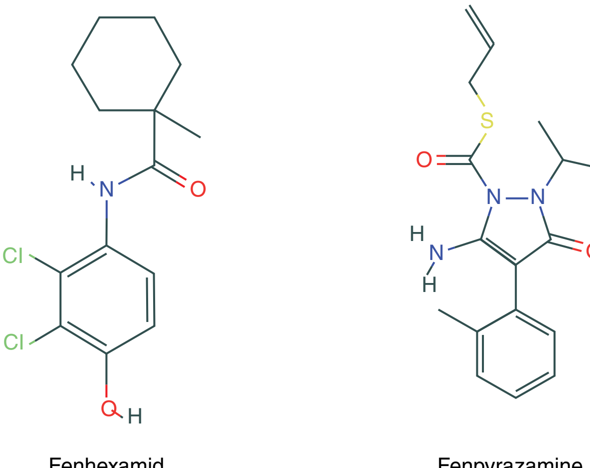

Fig. 5.21. G3 fungicides: the 3-keto reductase (*erg27*) inhibitors fenhexamid and fenpyrazamine. (Based on structures found at PubChem.)

Dimethomorph

Iprovalicarb

Benthiavalicarb

Valifenalate

Mandipropamid

Fig. 5.22. H5 cellulose biosynthesis inhibitors: dimethomorph, iprovalicarb, benthiavalicarb, valifenalate and mandipropamid. (Based on structures found at PubChem.)

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systemic movement. They operate by inhibiting germination of cystospores and sporangia, delaying elongation of hyphae and inhibiting sporulation.

I; Inhibition of melanin biosynthesis

The synthesis of the pigment melanin is important in fungal pathogenicity. The melanization of appressorial walls is essential for the development of infection hyphae and penetration of the host epidermis. Mutants of PYRIOR that do not contain melanin are not pathogenic. The discovery of tricyclazole initiated the development of chemicals displaying a novel MOA in pigmented ascomycetes, such as PYRIOR and various *Colletotrichum* species. Their inhibition of melanin synthesis provides excellent control of PYRIOR in rice and a significant share of the global market in fungicides (Motoyama and Yamaguchi, 2003).

Melanin biosynthesis in most fungi is via the DHN pathway. In this pathway, a ubiquitous polyketide synthase produces 1,3,6,8-tetrahydroxynaphthalene (Fig. 5.23). Further steps convert this to scytalone, to 1,3,8-trihydroxynaphthalene, to vermelone and, finally, to 1,8-dihydroxynaphthalene (DHN). The melanin biosynthesis inhibitor (MBI) group of fungicides is divided into I1 (MBI-R), which inhibit 1,3,6,8-tetrahydroxynaphthalene reductase (tricyclazole, pyroquilon and fthalide), and I2 (MBI-D), which inhibit the scytalone dehydratase (carpropamid, diclocymet and fenoxanil). The compounds inhibit the enzymes by substrate mimicry. Tolprocarb, a trifluoroethyl carbamate, inhibits the polyketide synthase step and this represents a new MOA (Fig 5.24).

The main targets of this group are PYRIOR and *Colletotrichum*. This limited spectrum can be explained by the critical role of the appressorium in cuticular penetration by these species, which seems to be solely due to turgor pressure. This places a huge premium on extremely tough appressorial cell walls. Any inhibition by these compounds appears to be sufficient to give control. On tricyclazole-treated rice, the early infection stages of PYRIOR (germination of conidia and formation of appressoria) are unaffected but the melanization of appressoria and the subsequent formation of the infection peg apparatus are inhibited, effectively protecting the plant from disease. Tricyclazole is readily taken up by leaves and roots of rice plants and translocated, predominantly acropetally. It is used in foliar applications and has mainly preventive activity.

Multi-site (M) and unknown (U) modes of action

Many older fungicides have proven to have multiple sites of action. This is generally associated with a poor toxicity profile and so many of these, such as arsenic and mercury compounds, have

Acetate → 1,3,6,8-Tetrahydroxynaphthalene → Scytalone → 1,3,8-Trihydroxynaphthalene → Vermelone → Melanin

Fig. 5.23. Melanin biosynthesis (dotted arrows indicate several steps).

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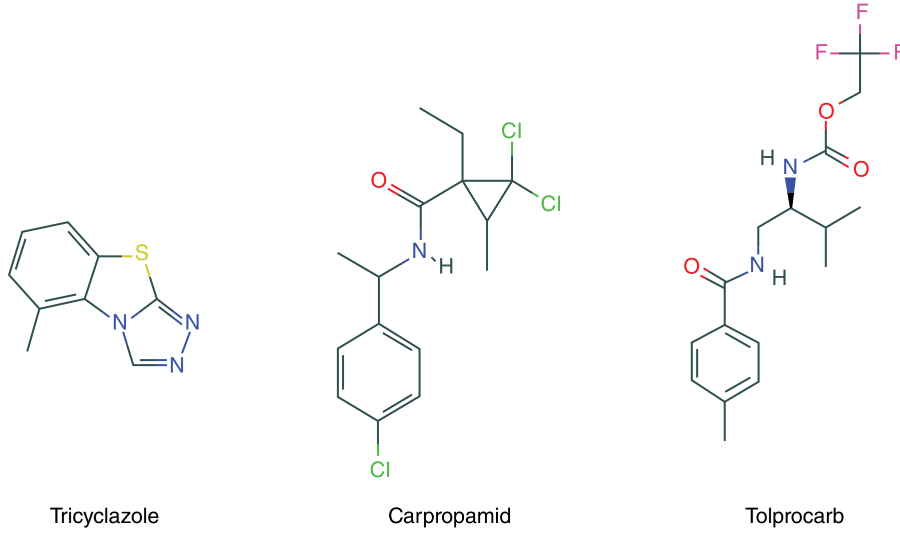

Fig. 5.24. Some I1/I2/I3 melanin biosynthesis fungicides: tricyclazole, carpropamid and tolprocarb. (Based on structures found at PubChem.)

been consigned to history. Others, such as copper and sulfur and many biologicals, are still widely used but as they are the mainstay of Organic disease control, they are dealt with in Chapter 6. Synthetic molecules with multiple sites of action are in the M category. Nowadays, it is necessary to determine the MOA before a new compound can be released. However, a few compounds were introduced prior to the rule and often the MOA has remained unclear. These are in the U class, although research continues and sometimes the MOA is clarified.

M03; Dithiocarbamates and relatives (electrophiles)

The discovery of the dithiocarbamate family of products in the 1930s and 1940s is usually accepted as initiating the period of organic synthesis of fungicides. As with most immobile protectants, dithiocarbamates are broad-spectrum fungicides with efficacy against basidiomycetes, ascomycetes and oomycetes. They are used as foliar, soil and seed treatments in fruit (VENTIN, *Taphrina deformans*), grapevine (PLASVI), vegetables (PHYTIN, BOTRCI, *Alternaria* spp., *Septoria* spp.) and sugarbeet (*Cercosporella beticola*); and are also used against downy mildew pathogens in tobacco (*Pseudoperonospora tabacina*) and hops (*Pseudoperonospora humuli*). The dithiocarbamates are inactive against the powdery mildews (*Erysiphales*).

Examples of the dithiocarbamates are ziram, zineb, ferbam and thiram (Fig. 5.25). Generally, dithiocarbamates are not phytotoxic but can induce damage in some crops in exceptional circumstances, for example in the use of mancozeb or zineb on zinc-sensitive plants.

M04; Phthalimides (electrophiles)

Phthalimides were introduced in 1952 with the announcement of captan and a close analogue, folpet (Fig. 5.26). They provide protectant control of a wide range of pathogens, are used extensively as sprays, root dips and seed treatments, and are useful in the control of damping-off of seedlings. They have been used to control PHYTIN, VENTIN, PLASVI and BOTRCI and many other foliar ascomycetes. They are inactive against members of the powdery mildews.

Captan and folpet preferentially react with enzyme sulfhydryl groups but may also attack amino groups and inhibit enzymes that do not contain sulfhydryl groups.

M05; Chloronitriles (unspecified mechanism)

Chlorothalonil is the sole chloronitrile fungicide and was introduced in the mid-1960s and is a

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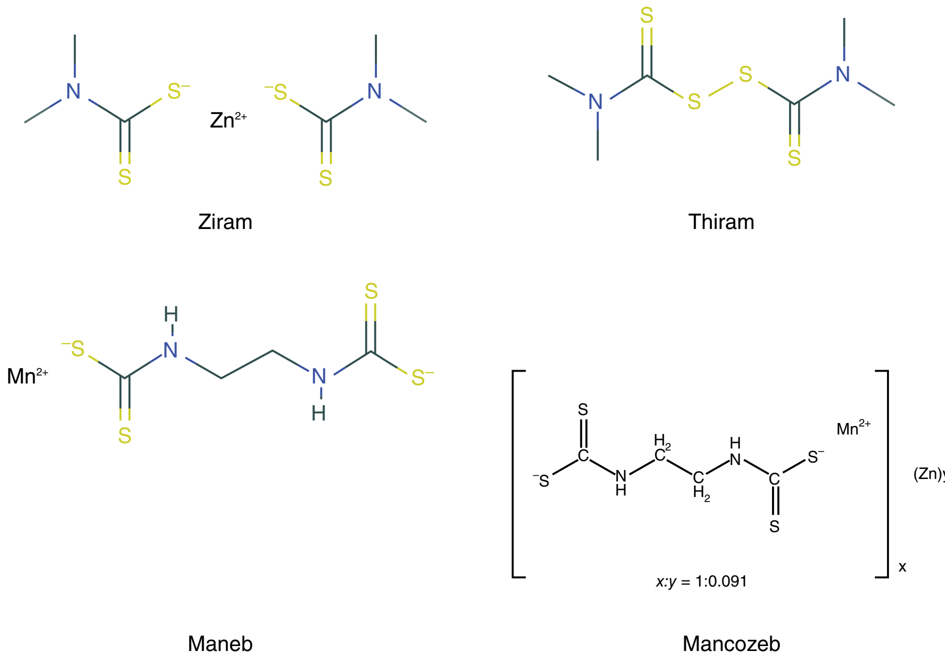

Fig. 5.25. M03 dithiocarbamate fungicides: ziram, thiram, maneb and mancozeb. (Based on structures found at PubChem.)

Captan

Folpet

Fig. 5.26. M04 phthalimide fungicides: captan and folpet. (Based on structures found at PubChem.)

major protectant fungicide. It is recommended mainly for use alone or in mixtures to control *Septoria* spp. in cereals, PHYTIN in potatoes and BOTRCI in vegetables and ornamentals, as well as finding uses in paints and preservatives (Fig. 5.27). Chlorothalonil binds to sulfhydryl and mercapto groups (Tillman *et al.*, 1973). It is widely used as a mixing or rotation partner with fungicides to improve the spectrum and for protection against fungicide resistance. However, it was deregistered in the EU in 2020 and its future is uncertain.

M06; Sulfamides (electrophiles)

The sulfamide tolylfluanid, introduced in 1971, is mainly used on apples, grapes and other perennial crops (Fig. 5.27). It controls VENTIN, PLASVI, PODOLE and UNCINE when used as a protectant. It reacts with –SH groups in proteins.

M07; Bis-guanidines (membrane disruptors, detergents)

The guanidine guazatine is a mixed product resulting from the reaction of polyamines

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Fig. 5.27. Miscellaneous multi-site and unknown MOA fungicides: M05, chlorothalonil; M06, tolylfluanid; M07 guazatine; U06, cyflufenamid; U12, dodine; U13, flutianil; U27, cymoxanil; U35, triazoxide. (Based on structures found at PubChem.)

(Fig. 5.27). It is used as a seed treatment in cereals for of control ascomycete fungi.

U06; Phenyl acetamides

Cyflufenamid is a phenyl acetamide introduced in 2002 (Fig. 5.27). It has preventive action for powdery mildew especially in cereals.

U12; Guanidines

Dodine is a guanidine introduced in 1957 (Fig. 5.27) and still in use as a systemic foliar protective fungicide for control of ascomycete diseases of perennial crops and vegetables. Recent work has indicated that disruption of mitochondrial and cell membranes is the main MOA (Schuster and Steinberg, 2020).

U13; Cyanomethylene thiazolidine

The cyanomethylene thiazolidine flutianil was introduced into the Japanese market in 2013 and 2014, to control powdery mildew on cucumber (Fig. 5.27). Cross-resistance with pyriofenone suggests a B6 MOA but this has not been confirmed (Miyamoto *et al.*, 2020).

U27; Cyanoacetamide-oxime

The cyanoacetamide-oxime cymoxanil is an extremely effective systemic fungicide with

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protectant and curative activity specifically against oomycete fungi (Fig. 5.27). Cymoxanil has important uses against PLASVI on grapevine and PHYTIN in which it is employed in a mixture with non-specific cell toxicant fungicides, for example mancozeb, as part of anti-resistance strategies to improve long-term activity and, through its curative activity, to extend the interval between sprays.

Cymoxanil is more effective against hyphal growth stages than early growth phases (the release of zoospores from sporangia and their germination). The compound inhibits nucleic acid and protein biosynthesis in *Phytophthora cinnamomi* and *Botrytis cinerea*, but it is likely that the activity is induced via an interaction with host metabolic processes.

U35; Benzotriazines

Triazoxide is a benzotriazine used only for control of seed-borne *Pyrenophora* diseases of barley (Fig. 5.27). It is not systemic. The basis of the limited spectrum has not been discovered.

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6

Biological Fungicides – Botanicals and Biocontrol Agents – and Basic Substances

Key Points

- Biological fungicides comprise a grouping of crop protection agents that excludes synthetic chemicals but includes BCAs, biorationals and botanicals.
- We also consider here 'basic substances', a group of simple chemicals with a long history of use in crop protection.
- Biological fungicides have multiple mechanisms of action and their interaction with the target pathogen is complex.
- Biological fungicides have low risk of resistance, low environmental impact and are compatible with multiple integrated pest management (IPM) strategies.
- Organic farming is governed by many different national and independent authorities. They typically permit biological fungicides and basic substances but exclude synthetic fungicides.

Introduction

A small but increasingly important sector of the fungicide market comprises a broad and diverse range of products that are collectively known as 'biologicals'. The defining attribute of these products is that they are made without the use of synthetic chemical procedures. The definition of 'synthetic' includes deliberate chemical modifications but excludes such procedures as solubilization, precipitation, extraction and stabilization. The biologicals group includes products designated or promoted as fertilizers, plant growth promoters, plant health promoters and for abiotic stress mitigation, but we are concerned here with 'biopesticides' and specifically 'biofungicides'. In addition to the true fungi, the targets of biofungicides include oomycetes, *Rhizaria* and even bacteria. Other groups of biopesticides are aimed at controlling insects, other arthropods and weeds. However, bioinsecticides are the largest group of biological pesticides to date.

Biofungicides fall into two classes. The first are applied as living organisms and are therefore also known as biocontrol agents or BCAs. All of these are microbes of various sorts. Their disease control effect depends on the growth and multiplication of the microbial inoculum. The second class is known as 'botanicals' or 'biochemicals' and sometimes as 'biorationals'. These are mostly extracts from plants and microorganisms (Kishore *et al*., 2007). The active ingredient/s are typically secondary metabolites – complex organic molecules produced by plants and microorganisms to defend themselves against other species.

We should also consider a group of products known as 'basic substances' (AHDB, 2017). These are a diverse group of products that includes inorganic compounds and products derived from animals and may have uses other than as fungicides.

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They have been used often for many centuries and so gained a reputation for efficacy and safety (Cook *et al.*, 1996). The features of these three types of fungicide considered here are summarized in Table 6.1.

All three groups of products are approved for use in the various forms of 'Organic/Biological/Ecological' agricultural cultivation although many of the products are also widely used in conventional agriculture. All the terms mentioned above – biologicals, BCAs, botanicals, biochemicals, low-risk substances and basic substances – are somewhat interchangeable, reflecting the diverse nature of the producers, users and regulators of these products (Marchand, 2017). However, there is a different bar, at least in the USA, for the registration of products marketed for 'plant health' rather than 'plant protection'.

The market for biological fungicides

The current size of the market for biofungicides (botanicals and BCAs) is small compared with the market for synthetics (*c.*$800 million versus $12.3 billion in 2013) but is growing much faster. Biofungicide sales grew by an annualized 16% between 2009 and 2014 compared with 5.5% for synthetics (Research and Markets, 2021). The number of biofungicide products has grown substantially over the past decades. Just 16 products were available in the UK in 2009 but by 2018 this had grown to 46. The area treated with biopesticides in the UK increased by 65% in just two years to 2015. However, the areas treated remain small compared with conventional fungicides. The biggest products were Serenade which was used on 1000 ha and Prestop used on 51 ha in the UK in 2015. This reflects their predominant use in horticulture and on protected crops. Basic substances have been in the marketplace for centuries but despite their age, their sales are still showing strong growth, along with the area devoted to Organic crops. Copper products alone have current sales of $500 million per annum (FAOSTAT).

There is a striking contrast between the two sectors in that innovation in the large synthetic market is dominated by just four companies (Syngenta, Bayer, BASF and Corteva) each with a global reach, whereas 20 companies account for two-thirds of the market in biologicals and fully 200 companies have introduced products into this sector. However, the market for biologicals is much more fragmented and many products are available only in a few regions.

The diverse nature of the products is reflected in the observation that a major proportion of the research leading to the discovery of biologicals started in universities and in small, start-up companies. The major agrochemical companies are also very active in this field and undertake substantial in-house research and development. However, they are now supplementing their in-house capabilities by acquiring the smaller biological companies. Merger and acquisition deals exceeded $2.5 billion by 2010. Basic substances are produced and marketed by many companies, most of which also sell generic synthetic pesticides.

History

The use of inorganic chemicals – such as compounds of copper, sulfur and mercury – to help control disease goes back to the 18th and 19th

Table 6.1. Biological fungicides and basic substances. (Authors' own table.)

Type	Definition	Examples	Main modes of action
Biocontrol agents (BCAs)	Living organisms; viruses, bacteria and fungi	Contans WG *Coniothyrium minitans 91-08*	Competition, hyperparasite, antibiosis, plant defence stimulation
Botanicals	Organic chemical compounds directly extracted from plants and fungi	Regalia	Contact toxin, defence stimulation
Basic substances	Inorganic compounds or household products with a long history of use	Copper, sulfur	Broad-spectrum biocides; denature enzymes, interfere with the electron transport system and disrupt cellular membranes

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centuries with the work of Tillet and Millardet (see 'The History of Fungicide Use' section, Chapter 1, this volume). Some of these compounds remain in use to this day, now called 'basic substances', while others have been deemed to be too toxic.

Research and development in the field of biofungicides remained largely silent through the era of the contact fungicides up to the 1970s and the start of the era of systemic synthetic chemicals. The environmental movement grew following the publication of *Silent Spring* in 1962 (Carson, 1962), as did the market for 'Organic' produce, at least in richer and more urban regions. Evolved resistance to fungicides was first recognized in the 1960s and has continued to grow to this day. All these factors combined to increase the demand for crop protection methods that promised to be more sustainable in use and induce less damage to the environment, growers and consumers. With the widespread rejection of genetically modified (GM) crops since 1998, the search for biofungicides increased substantially. A critical milestone was the adoption in 2009 of the EU's 'Sustainable Use Directive 2009/128/EC'. This directive substantially increased the difficulty of registering synthetic crop protection products and provided a relatively fast-track registration process for biologicals. As a result of these regulatory and market changes, the cost of bringing a synthetic chemical to market is now estimated to be $256 million and to take 10–15 years whereas for a biopesticide the estimates range from $3 million to $6 million and only 4–5 years. It is no surprise therefore that there are now more biopesticides than synthetic chemicals awaiting registration by the EU. The EU has continued its support of biopesticides with the publication of its Green Deal. This goal for 2030 is to make 25% of the EU agricultural area Organic and reduce synthetic pesticide use by 50%.

Discovery and Development of Biopesticides

Biocontrol agents

The concept underlying the use of BCAs is that the growth of a pathogenic organism can be inhibited by the simultaneous growth of another organism, resulting in reduced disease incidence and higher yield and/or quality of the crop. In ideal cases, the BCA forms a stable population that controls the pathogen such that the levels of disease are within manageable limits over multiple crop growing cycles. In practice, most uses of BCAs require at least one application of the agent during the growing season and often several. The main features of currently available BCAs are summarized in Table 6.2.

The discovery of BCAs starts with the isolation of test microorganisms, fungi, bacteria and viruses. The microorganisms are often sourced from existing culture collections, but they are more often novel isolates collected from soil or infected plant material. A good source of organisms has been soils in which disease unexpectedly did not occur, so-called 'suppressive soils'. The next challenge is to maintain the organism as a pure culture in a stable state. Considerable expertise and expenditure are required to achieve these initial steps as each of the bacterial and fungal species is likely to have specific growth requirements. One of the larger companies in this area, Marrone Bio Innovations, reported in 2014 that they were screening more than 40,000 samples and 18,000 isolates each year.

To screen for biocontrol activity, the microorganism must be tested against the target pathogen and disease. This can take place *in vivo*, where both organisms are grown in agar media. Inhibition of the growth of the pathogen is a sign of a positive lead. Furthermore, direct observation of the agar plate can give clues as to the MOA. The next step is to spray the biocontrol lead on to plants either before or during infection. This can involve seeds, seedlings, adult plants or leaf discs. These are complicated processes so the number of organisms that can be tested is typically much lower than when synthetic chemicals are used.

Having verified the biological activity, the next steps are to determine the spectrum of activity and the longevity of the protection. Unless the organism has been obtained from a culture collection, it will be necessary to carry out a formal and complete identification. This will require light and scanning electron microscopy studies complemented by molecular genetics. Nowadays full genome sequencing is a cost-effective way to determine the identity of the isolate. Such studies will be required if the organism proceeds to patent protection.

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Table 6.2. Biocontrol fungicides for crops. (Authors' own table.)

Biological control agent	Product	Company	Target pathogen/disease	Crop	Mode of action
Agrobacterium radiobacter K1026	Nogal	BASF	*Agrobacterium tumefaciens*	Walnut, pome fruit, berries, flower crops, grapes	Competition
Agrobacterium radiobacter K84	Galltrol-A	AgBioChem Inc.	*Agrobacterium tumefaciens*	Walnut, pome fruit	Competition
Ampelomyces quisqualis M-10	AQ 10	CBC, Belchim Crop Protection Ltd and Fargro Ltd	Powdery mildews	Wide range of protected edible fruit and vegetables, and ornamentals (EAMU)	Hyperparasite
Aspergillus flavus AF36 and	*Aspergillus flavus* AF36	Arizona Cotton Research and Protection Council	*Aspergillus* spp.	Cotton, groundnut	Competition – strains do not make aflatoxin
Aspergillus flavus NRRL 21882	Alfal-Guard GR	Syngenta			
Aureobasidium pullulans DSM 1490 and 14941 (mixture of isolates)	Botector Boni Protect Blossom Protect	Bio-ferm GmbH	*Erwinia*, BOTRCI, PENIEX, ALTEAL	Pome, stone fruits and grapes	Competition
Bacillus amyloliquefaciens D747	Double Nickel	Certis USA	All diseases	Greenhouse ornamentals, vegetables and herbs	Antibiosis (iturins)
	RhizoVital 42	Verdera Oy and Fargro Ltd			
	Double Nickel BA	Andermatt Biocontrol AG			
Bacillus licheniformis SB3086	Roots EcoGuard	Lebanon Turf	Wide range but especially dollar spot, anthracnose	Turf, ornamentals, forestry, etc.	Antibiosis
Bacillus pumilus QST 2808	Cease Serenade Rhapsody	BioWorks BioWorks Bayer	BOTRCI, *Erwinia*, VENTIN, *Erysiphe* spp., *Sclerotinia* spp., *Monilinia* spp.	Roots of many crops, potatoes	Competition
Bacillus subtilis subsp. *amyloliquefaciens* FZB24	Taegro	Novozyme	PHYTIN, *Fusarium* spp., RHIZSO	Tomato, ornamentals lettuce, potato, wheat	Plant defence stimulation
Candida oleophila O	NEXY0101	Bionext sprl	BOTRCI, PENIEX	Pome	Competition
Phage against *Clavibacter michiganensis* and *Bacillus subtilis* subsp. *amyloliquefaciens* FZB24	Agriphage	Omnilytics	*Clavibacter michiganensis*, *Pseudomonas syringae*, *Xanthomonas campestris*	Tomato	Lyses bacteria

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Coniothyrium minitans 91-08	Contans WG	Bayer	*Sclerotinia* spp.	Many broadleaf species	Hyperparasite of sclerotia
Gliocladium catenulatum J1446 (*Clonostachys rosea*)	Prestop	Verdera Oy, Fargro Ltd	*Botrytis* spp., many soil fungi oomycetes	Soft fruit, lettuce	Competition; antibiosis
Gliocladium virens GL-21 (*Trichoderma*)	SoilGard	Certis	*Pythium*, *Phytophthora* spp., *Fusarium* spp., *Sclerotinia* spp., RHIZSO	Melon, strawberry	Antibiosis – gliotoxin; hyperparasite; competition
Pseudomonas chlororaphis MA342	Cerall Cedomon	Lantmannen BioAgri AB	*Pyrenophora* spp., *Tilletia* spp., *Ustilago* spp., *Fusarium* spp., *Bipolaris* spp., *Microdochium*	Barley, oats germination	Antibiosis; hyperparasite; competition
Pseudomonas fluorescens A506	BlightBan A506	NuFarm	*Erwinia amylovora*, BOTRCI	Pome, lucerne, almond, potato, grape	Frost protection
Pseudomonas sp. DSMZ 13134	Proradix	Sourcon Padena	PHYTIN, RHIZSO, *Helminthosporium solani*, *Erwinia carotovora*	*Solanaceae*, cucurbits, lettuce, ornamentals	Sequestration of iron; resistance inductions
Pseudomonas syringae pv. *tomato* phage	Agriphage	Omnilytics	*Pseudomonas syringae* pv. *tomato*	Tomato, pepper	Lyses bacteria
Pseudomonas syringae ESC11	Bio-Save 11LP	Jet Harvest Solutions	BOTRCI, *Mucor* spp., *Peronospora* spp., *Rhizopus*	Pome, sweet potato	Competition
Pythium oligandrum M1	Polyversum	Biopreparity	BOTRCI, ALTEAL, LEPTMA, SCLESC, *Verticilllium* spp.	Crucifer and sunflower	Antibiosis; hyperparasite; competition; resistance induction
Streptomyces griseoviridis K61	Mycostop	Verdera Oy	BOTRCI, FUSAOX, RHIZSO, *Pythium*	Herbs, ornamentals	Competition, antibiosis
Streptomyces lydicus WYEC 108	Actinovate AG	Novozymes	Various root pathogens, e.g. *Pythium*, *Rhizoctonia*, *Fusarium*, *Phytophthora*; various foliar pathogens, e.g. powdery mildew, *Botrytis* spp. and others	Greenhouse ornamentals, vegetables and herbs	Competition; antibiosis

Continued

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Table 6.2. Continued.

Biological control agent	Product	Company	Target pathogen/disease	Crop	Mode of action
Trichoderma spp.	Bio-Tam	Bayer	Damping-off fungi, Eutypa dieback	Many; for grape and root diseases in greenhouse ornamentals	Competition; antibiosis; mycoparasite
	T34 Biocontrol	Biocontrol Technologies			
	Xedavir	XEDA			
	Esquire WP	Bayer			
	Binab TF WP	BINAB Bio-Innovation			
	Tenet	Agrimm Technologies			
	Incept	Syngenta			
	Eco-77	Madumbi Sustainable Gariculture			
	Trianum	Koppert			
	PlantShield HC	BioWorks			
	T-22 HC	BioWorks			
	RootShield	BioWorks			
	Ecosom-TV	AgriLife			
Phage against *Xanthomonas campestris* pv. *vesicatoria*	Agriphage	Omnilytics	*Xanthomonas campestris* pv. *vesicatoria*	Tomato	Lysis

EAMU, Extension of Authorisation for Minor Use.

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BCAs must be able to at least maintain and hopefully grow their population sizes during crop growth. That way, disease control is maintained. A key step is the determination of whether the inhibitory activity is due the action of a secreted compound (either a secondary metabolite or an enzyme) or requires the presence and growth of the microorganism to achieve disease control via niche competition or mycoparasitism. If the former, the active compound can be purified, identified and synthesized. An active compound could then be used directly as a botanical (see 'Botanicals' subsection below) or as a lead compound for the design of a family of synthetic derivatives, as was the case for strobilurins (see 'C3; Complex III; cytochrome bc1 (ubiquinol oxidase) at Qo site (*Cytb* gene); QoI' section in Chapter 5, this volume).

The toxicity profile of the organism and its secreted products must be tested and shown to be within acceptable limits. If they lack obvious toxic effects, such as neurotoxicity or endocrine disruption, they may be deemed 'low-risk substances' in the EU and thus subject to much more benign regulations than is the case for synthetic fungicides (Marchand, 2017). However, the regulatory hurdles are still onerous as they are based on hazard (the ability of a product to cause harm) rather than risk (the likelihood of a product causing harm).

The biggest challenge in the production of a BCA is the growth of a microorganism such that it can be formulated into a safe, stable and active product in a cost-effective manner. In the early stages of development, small shake flasks may be sufficient, but the final production scale is likely to need large fermenters of at least 100 litres. Considerable effort is needed to find suitable fermentation, processing and formulation formats (Schisler *et al.*, 2004). Quality control studies are needed together with toxicity studies on non-target organisms. However, minimum residues studies are not needed if the product is deemed safe. The final stages are regulatory approval and marketing (Mathre *et al.*, 1999; Leahy *et al.*, 2014).

Botanicals

Botanicals or biorationals are terms used for compounds extracted from living organisms without any chemical modification and used to control pests. Nearly all botanicals have been obtained from plants and microorganisms and emerge from the studies of the myriad of ethnobotanists and microbiologists operating in academic and industrial laboratories around the globe. In the case of plants, a fertile source is the medicinal herbs used by indigenous civilizations all around the world. A key factor is to understand how to grow the plant and make the extract to obtain a useful biological activity (Fennell *et al.*, 2004).

A crude extract, either aqueous or in simple solvents like ethanol or methanol, is then tested for a range of biological activities not only against phytopathogens but also animal pathogens and human cancer cell lines. Initial screens are usually *in vivo* tests but would also include *in planta* screens against key diseases (as described in 'Screening for Fungicide Leads' section, Chapter 4, this volume). The structure of any active ingredient would be determined by purification and identification using mass spectrometry, nuclear magnetic resonance spectroscopy (NMR) and X-ray crystallography. Preliminary toxicity tests are carried out together with tests to determine the activity spectrum and whether the MOA is novel.

So far, all these steps are the same for conventional and biological fungicides. The key difference in a biological fungicide is that the natural product can be extracted from biological sources in a cost-effective manner and the final product has adequate activity, spectrum and stability for direct use in the field (Table 6.3). If so, the next steps are to optimize the production of the product, develop a formulation, obtain regulatory approval and plan the marketing campaign.

An example of the development of a biofungicide is Regalia, marketed by Marrone Bio Innovations. Giant knotweed (*Reynoutria sachalinesis*) had been researched as a source of bioactive molecules since the 1990s. Marrone developed a formulation based on an ethanolic extract of dried plant material. The extract was found to be mainly the anthroquinones emodin and physcion (Fig. 6.1), but also to contain other molecules. These metabolites are found in a range of plant species where they were thought to provide antifungal and herbivore defence. The spectrum of the product was investigated and found to be much wider than previously suspected and purported to include BOTRCI, powdery and downy

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Table 6.3. Botanicals patented and marketed for disease control. (Authors' own table.)

Active ingredient	Product	Company	Target pathogen/disease	Crop	Mode of action
Eugenol, geraniol	Mevalone	Eden Research PLC	BOTRCI	Grapes	Contact toxin
Fennel oil	Fenicur	Andermatt Biocontrol AG	Powdery mildew spp.	Cucurbits, *Solanaceae*, cane fruit	Unknown
Laminarin	Vacciplant	Arysta LifeScience	Many	Many	Induction of defence
Neem oil	Trilogy	Certis	Many	Many	Inhibition of fungal germination
Orange oil	Prev-AM	Oro Agri	Powdery mildews	Fruits	Contact toxin
Extract of *Reynoutria sachalinensis* (Lewis *et al*., 2016)	Regalia	Marrone Bio Innovations	Foliar diseases, e.g. anthracnose, bacterial leaf spots, BOTRCI, ALTESO, PHYTIN, downy mildew, fungal leaf spots, powdery mildew	Cucurbits, *Solanaceae*, pome fruit, greenhouse ornamentals, vegetables and herbs	Induction of defence

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mildews and various bacterial diseases. It also has activity when applied as a seed dressing to control damping-off diseases. This broad spectrum suggested that the MOA was via induction of plant defence, and this turned out to be the case. Regalia induces the accumulation of reactive oxygen species, the expression of PR-proteins and the development of physical barriers to penetration. As such, Regalia can be compared with the other products in the FRAC class P. A clear advantage for this group is that resistance is not expected to occur.

Registration was given by the US Environmental Protection Agency (EPA) in 2009 just one year after submission. However, gaining clearance in the EU proved difficult. Extracts from biological sources often contain a mixture which varies from batch to batch. Furthermore, because of the toxicity profile, the EU indicated that lower rates would be needed to achieve registration. At these lower rates, the efficacy in disease prevention was found to be marginal even though crop yield, quality and drought tolerance were enhanced. It was therefore rebranded as a growth promoter, but as different rules apply, further investment was needed to complete the application dossier.

Fig. 6.1. Active ingredients of Regalia; R = H is emodin; R = CHO_3 is physcion. (Data from PubChem.)

Regalia can be applied to seeds, as a dip for transplanted seedlings into a furrow or in irrigation water and is best suited to horticultural uses. The activity is not systemic but is translaminar and thus Regalia needs to be reapplied at frequent intervals. At the low rates allowed by the EU, the efficacy was low but could be improved by the addition of low levels of conventional fungicides. The clear synergism is likely to be due to the weakened state of the pathogens after plant defences are induced making them more susceptible to the direct inhibitory effects of the conventional fungicide. Although such co-use has a great deal to recommend it, the addition of strobilurin or triazole fungicides would not be acceptable to Organic producers.

Basic Substances

A small number of chemical compounds are defined in EU legislation as 'Basic Substances' and some of these are used to control diseases (Table 6.4). These compounds have been known and widely used for centuries and so they are exempt from many of the regulations surrounding the use of novel biological and conventional fungicides. They are included in this section as most are allowed in Organic agriculture. Although they are widely and generally regarded as safe (GRAS), it is not always clear that they would pass the stringent regulations now in force for pesticide registration.

Table 6.4. Basic (minimum risk) substances used to control diseases. (Authors' own table.)

Basic substance	Target disease(s)	Crops
Lecithin	Powdery mildews; PHYTIN	Salad crops, tomato
Copper salts	Downy mildews, bacterial diseases	Fruits, vegetables, perennial crops
Lime sulfur	Downy mildew, anthracnose, black rot, *Exobasidium*, phomopsis, leaf curl	Grapevine, berries, stone fruit
Calcium hydroxide	*Neonectria*	Pome and stone fruit
Salix cortex		Pome and stone fruit
Vinegar	Bunts and smuts, various foliar bacterial and fungal disease	Wheat, barley, vegetables, ornamentals and trees
Whey	Powdery mildew	Cucurbits
Equisetum arvense (horsetail) extract	Scab and mildew	Grapevine, fruit trees, cucumber, tomato
Sunflower oil; seed oils (sunflower, jojoba, etc.)	Powdery mildew	Vegetables, ornamentals

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In the USA, these products are designated by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Sec. 25(b). This designation was criteria set by 40 CFR 152.25(f) and exempted from federal regulation. In the USA, a 'Minimum Risk' product must meet the following six conditions:

1. The product's active ingredients must only be those that are listed in 40 CFR 152.25(f)(1).
2. The product's inert ingredients may only be those that have been classified by the EPA as listed in 40 CFR 152.25(f) and commonly consumed food commodities, animal feed items and edible fats and oils as described in 40 CFR 180.950(a), (b) and (c); and certain chemical substances listed under 40 CFR 180.950(e).
3. All of the ingredients (both active and inert) must be listed on the label. The active ingredient(s) must be listed by label display name and percentage by weight. Each inert ingredient must be listed by label display name.
4. The product must not bear claims either to control or mitigate organisms that pose a threat to human health, or insects or rodents carrying specific diseases.
5. The name of the producer, or company for whom the product was produced, and the company's contact information must be displayed prominently on the product label.
6. The label cannot include any false or misleading statements (40 CFR 152.25(f)).

Sulfur fungicides

The oldest pesticide, sulfur, occurs naturally throughout the world as sulfites (e.g. pyrite and chalcocite, FeS_2 and CuS_2), sulfates (e.g. gypsum) and in its bright yellow, elemental state as pure crystals. It is associated with volcanoes (active and extinct) and hot mineral springs (Beckerman, 2008). The fungicidal properties of sulfur were known even before an understanding of the role of microorganisms in plant disease was established. In the early 1800s, elemental sulfur was used to protect peaches, grapes and roses from their respective powdery mildews. Lime sulfur for pesticide use was first described in 1802 in England (Tweedy, 1969). There have been few modifications or improvements to sulfur as a fungicide, despite all the work that has gone into the formulation and application equipment for other pesticides.

The history of sulfur as a fungicide coincided with the recognition that it was often phytotoxic, especially in warmer climates. In apple, there are reports of sulfur-sun scald on the fruit, and even the prevention of fruit set due to the toxicity of sulfur to pollen germination. In other fruit crops, sulfur causes defoliation in some varieties of gooseberries; damage and even death to cucurbits and raspberries; and insoluble residues often could not be removed from other fruit, adding to corrosion in the canning (preservation) process. High levels of sulfur inhibit yeasts, preventing fermentation and resulting in off-flavours to wines. Despite the issues, when used and timed correctly, sulfur is one of the safest fungicides (and insecticides) available for crop protection.

Copper and other metals

Metals play an important role in the life cycle of all organisms, during germination, growth, metabolism and reproduction. Some metals are essential for life (e.g. calcium, copper, cobalt, iron, potassium, magnesium, manganese, sodium, nickel and zinc) while others are found in organisms but with no known function (e.g. aluminium, silver, gold, caesium, cadmium, mercury, rubidium and lead). As with all toxins, it is the dose that makes the poison and metals (regardless of being essential or non-essential) are all toxic above a certain concentration (Johnson, 1935; Beckerman, 2008). This concentration is dependent upon the type of organism, the environment and the metal itself. The toxicity of metals is due to their ability to displace and/or replace the desired metal ions from enzymes and other biomolecules, along with denaturation and inactivation of enzymes and proteins, as well as disruption of membrane integrity of the cell and its organelles (Gadd, 1994; Borkow and Gabbay, 2005).

The antimicrobial properties of metals have been recognized for millennia. The ancient Persians, and later the ancient Phoenicians, Greeks, Romans and Egyptians used vessels and amphorae made of copper and silver to preserve and disinfect food and drink. This practice was continued (despite the heavy metal poisoning) by European settlers to North America, who added silver coins into containers to preserve water, wine,

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milk and vinegar (Lemire *et al*., 2013). Silver is currently used as a drinking-water disinfectant but is too expensive for use in agriculture. Other metals such as mercury were used in crop protection and in medicine but are now deemed too toxic for routine use. Hence the only metal used in crop protection to any extent is copper.

Copper naturally occurs as a usable metallic form, unlike most other metals, permitting its early adoption and use. Copper is one of the oldest compounds used in plant protection, credited to Millardet in 1882, who observed that a mixture of copper sulfate ($CuSO_4$, bluestone) and slaked lime ($Ca(OH)_2$) applied to grapes to deter against pilfering also worked against grape downy mildew – this was marketed as the 'Bordeaux mixture'. This 'discovery' occurred 75 years after Prevost used copper sulfate for treatment of wheat seeds against smut. This process was improved by Dreisch in 1873, who used a limewater bath after treatment with copper sulfate. In a matter of years other formulations were developed, for example 'Burgundy Bordeaux mixture' which consists of copper sulfate and sodium carbonate (Na_2CO_3).

Not surprisingly, the use of copper from copper sulfate expanded to other fixed copper chemistries and currently includes formulations of basic copper sulfates (green and blue coppers), basic copper chlorides (blue to bluish green), copper oxides (red coppers) and tribasic copper sulfate (green and blue coppers), copper–ammonium complexes (a dark blue aqueous complex of copper and ammonia) and copper octanoate (copper soap). Although older formulations contained high levels of elemental copper in wettable powders, many modern formulations contain between 1.8 and 50% Cu (w/w). As a result, application rates vary accordingly. In addition to wettable powders, modern copper fungicides are formulated as water-dispersible granules (WDG), liquid flowable (F) suspensions or aqueous liquids (Table 6.5).

RECENT TRENDS IN COPPER USE. Copper fungicide use declined in North America and Europe in the 1950s after the development of synthetic fungicides. However, use in Central and South America intensified in attempts to control MYCOFI on bananas. Tall plants (12 m) and frequent rainfall meant that copper was applied 20–30 times a year. The workers became known as 'pericos', the Spanish word for parakeet, due to the blue-green colour that coated workers and their clothing (Marquardt, 2002). The health issues that resulted from applications like these, in the absence of personal protective equipment (PPE), were considerable. Although scientists often focus on risk assessment as a linear process (risk identification, analysis, forecasting and communication), it is important to remember that social movements, including worker protests, often influence risk perception and assessment (Barraza *et al*., 2013) and what is acceptable use. Human health, environmental impact, along with efficacy issues contributed to a decline in the use of copper. In contrast to North America, copper is still widely used in the EU and not only on Organic crops. However, the growing realization of the toxicity and persistence has led to it being under intense review (Fig. 6.2). Some countries, such as Sweden, have banned all further use of copper.

Mode of Action of Biofungicides

Biological fungicides have a broad range of MOAs. Each MOA predicts the spectrum of the product, the longevity of effect and the risk of resistance. The MOAs of biofungicides are:

1. Competition or exclusion.
2. Antibiosis.
3. Hyperparasitism.
4. Defence induction.
5. General biocidal activity.
6. Miscellaneous.

Competition or exclusion

The commonest MOA claimed for BCAs is niche competition, sometimes referred to as exclusion when the biological control organism impacts the target pathogen's growth, activity and/or reproduction. 'Competition' is used when the organism can be applied *after* the pathogen has infected. 'Exclusion' is the preferred term when it must be applied *before* the pathogen is present. None the less, the terms are somewhat overlapping.

Fifteen biofungicides cite competition as their sole or partial MOA. This concept emerges from the realization that all plant tissues harbour not

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Table 6.5. A comparison of copper fungicides, formulations and particle size. (Based on information derived from Creek *et al*., 2017 and EPA, 2009.)

Product name	AI	Formula	CAS Number	Cu (g/kg)	Mean particle size (μm)	Amount of AI (%, w/w)	MCE[a] (%, w/w)	Manufacturer/ distributor
Badge X2	Copper oxychloride + copper hydroxide	$3Cu(OH)_2CuCl_2$	1332-65-6 + 20427-59-2	Not disclosed	8.4	23.8 + 21.5	28.2	Mineral Research & Development
Blue Shield DF	Copper hydroxide	$Cu(OH)_4$	20427-59-2	500	2.5	50	76.8	Bayer Crop Science
Bordeaux WG	Tribasic copper sulfate	$CuSO_43Cu(OH)_5$	1344-73-9	200	1.9	Not disclosed	Not disclosed	Melpat International
Champ Dry Prill WG	Copper hydroxide	$Cu(OH)_3$	20427-59-3	375	0.15	37.5	24.4	Nufarm
COCS	Copper oxychloride sulfate	$Cu_4(OH)6(SO_4)$	8012-69-9	Not disclosed	1.8–3.0	51.3	87.0	Loveland Products, USA
Copper Count N (Soluble)	Copper diammonium diacetate complex	Copper ammonium complex	16828-95-8	Not disclosed	Not disclosed	8	31.4	Chemical Specialties, Inc., USA
Coppox WG	Copper oxychloride	$3Cu(OH)_2CuCl_2$	1332-40-7	500	1.4	50	84	Melpat International
Cueva	Copper octanoate	$C_8H_{16}O_2Cu$	20543-04-8	Not disclosed	Not disclosed	10	1.8	Certis, USA
Cuprofix Disperse WG	Tribasic copper sulfate	$CuSO_43Cu(OH)_2$	1344-73-6	200	3	40	71.1	Nufarm
MasterCop	Copper sulfate pentahydrate	$CuSO_4{\cdot}5H_2O$	7758-99-8	213.6 g/l (equivalent to 60 g Cu/l)	Not disclosed	21.5	5.4	Adama
Kocide Blue Xtra	Copper hydroxide	$Cu(OH)_2$	20427-59-2	350	0.1–1	35.0	53.8	DuPont
Nordox WG	Cuprous oxide	Cu_2O	1317-38-0	750	1	83.9	75	Tanuki
Tribase Blue	Tribasic copper sulfate	$CuSO_43Cu(OH)_3$	1344-73-7	190	0.7	Not disclosed	Not disclosed	Nufarm

AI, active ingredient; MCE, metallic copper equivalent.

[a]MCE is a commonly used measure of the quantity of copper in fungicides and differs from the amount of AI because not all the copper in a formulation is actually available.

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Fig. 6.2. Characteristic symptoms of phytotoxicity due to copper-based antimicrobial compounds on citrus fruit surface. Such a visible damage on fruit surface markedly reduces the aesthetic value of the fruits thereby compromising their marketability. (From Lamichhane *et al.*, 2018, with permission.)

only plant pathogens but also a diverse array of benign microorganisms (Andrews and Harris, 2000; Berendsen *et al.*, 2012). The study of the plant microbiome has mushroomed in recent years. These putatively benign organisms are in competition with pathogens for nutrients and possibly even for oxygen and water. Other crop protection methods and especially synthetic fungicides can denude the plant of protective microorganisms, leaving the plant vulnerable to attack.

The essential characteristic of a competitive BCA is that it should efficiently colonize the available space and nutrient sources that would otherwise be available for the pathogen. An obvious starting point for such a BCA is to screen for close relatives of the pathogen that lack a key pathogenicity factor so that they do not cause disease themselves. Such close relatives are likely to have similar niche requirements and growth characteristics. Examples include *Agrobacterium* strains that do not induce galls and strains of fungi such as *Aspergillus* and *Fusarium* that do not produce damaging mycotoxins. Niche-competitive BCAs are thought to be low risk for the development of resistance.

Antibiosis

A large group of BCAs exerts their effect by producing molecules that interfere with the development of pathogens. A key test for this MOA is that the isolated biomolecule should be able to inhibit the disease. Attractive features of this type of BCA are that the antifungal/antibiotic is produced without the need for chemical factories or fermenters, and the effect is produced exclusively where it is needed and extends for the life span of the BCA.

In many cases the identity of the molecule is unknown (or at least undeclared), but the described examples can be divided broadly into small-molecular-weight secondary metabolites and proteins. Gliotoxin, produced by *Gliocladium virens*, was discovered in the 1930s. The metabolite has been shown to have a variety of biological activities but evidence that it is a key component of the biocontrol activity is still controversial (Scharf *et al.*, 2016; Sherkhane *et al.*, 2017). Random mutants lacking gliotoxin had reduced biocontrol activity. Disruption of the gene cluster required for the biosynthesis has been achieved. This opens up the possibility of finalizing its role.

Hyperparasitism

Like nearly all organisms, the pathogens that cause diseases of plants are themselves subject to diseases. The pathogens that cause diseases of pathogens are called hyperparasites but if the host and host are both fungi, they can also be termed mycoparasites.

Some mycoparasites resemble biotrophic phytopathogens in having narrow host ranges and causing only slow death of the host fungus. This applies to *Ampelomyces* which parasitize the hyphae of powdery mildew species and *Coniothyrium minitans* that colonizes the sclerotia of *Sclerotinia*. The *Trichoderma* group, on the other hand, are free-living organisms with broad host ranges and as such bear more resemblance to necrotrophic phytopathogens (see 'Phytopathogenic lifestyles; biotrophs, necrotrophs and hemibiotrophs' section in Chapter 2, this volume). The various *Trichoderma* preparations utilize a range of MOAs (Howell, 2003), including antibiosis and competition, but there is strong evidence of direct parasitism involving invasion of host hyphae (Druzhinina *et al.*, 2011).

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Defence induction

Defence in plants can be summarized as a response to specific substances that induce the expression of a generalized defence response (see 'Avirulence genes, PAMPs, MAMPs and effectors' section in Chapter 2, this volume). Hence infection by a non-pathogenic microbe can often induce resistance to pathogenic species, either locally or in distant (systemic) tissue. This MOA has been exploited in several BCAs and botanicals. The Novozyme product Taegro, based on *Bacillus subtilis* subsp. *amyloliquefaciens* FZB24, colonizes plant roots and induces defence responses that protect against damping-off pathogens (Jacobsen *et al.*, 2004). Isolated products such as Laminarin and Regalia also have this MOA. Like the synthetic molecules in FRAC class P, resistance is not expected to be an issue.

Biocidal activity

Copper functions as a broad-spectrum biocide (it is also effective against bacteria, algae, molluscs and many microorganisms) and denatures proteins and enzymes, interferes with the electron transport system and disrupts cellular membranes (Fleming and Trevors, 1989). By itself, copper sulfate is highly phytotoxic and kills growing plant tissue. The inclusion of slaked lime as a safener results in colloids of copper hydroxide, creating 'fixed copper' that prevents plant rapid absorption and phytotoxicity. This feature also provides better residual activity against diseases than non-fixed copper fungicides.

Lime sulfur also has a direct biocidal activity. When diluted to 2% (w/w) for application, it has a pH of 10.0 and releases small amounts of hydrogen sulfide (H_2S) gas (which provides the rotten egg odour). This H_2S gas permeates the fungal membrane and interferes with multiple targets of mitochondrial respiration, while providing broad-spectrum efficacy (Tweedy, 1981; Beffa *et al.*, 1987; Beffa, 1993a,b). This non-target efficacy can translate into phytotoxicity if leaf wetness and/or high relative humidity occur during an application (Subhash, 1988; Tate *et al.*, 2000).

Miscellaneous modes of action

There are several other MOAs represented by just one current product. The product Proradix comprises a *Pseudomonas* strain that secretes a siderophore molecule that adsorbs all the available iron in the vicinity of the root. Iron is an essential element required for fungal respiration and other biochemical functions. The lack of available iron inhibits the growth of damping off caused by RHIZSO. This type of control mechanism needs to balance the micronutrient needs of the plant with the needs of the pathogen.

The active ingredient of BlightBan A506 is an isolate of *Pseudomonas fluorescens* that displaces other bacterial species that promote the formation of ice crystals that might damage flowers and buds. The frost damage promotes infection by *Erwinia* and BOTRCI in crops such as apples and grapes. A506 was isolated from pears and replaced an earlier product in which the gene for the ice nucleation protein was deleted using genetic modification methods (Stockwell *et al.*, 2010). The GM ice-minus bacterium was called Frostban and was the first GM product destined for release into the environment but was never marketed following widespread protests.

Using Biological Fungicides

Biocontrol agents

Methods for the application of BCAs differ substantially from those for conventional fungicides and indeed botanicals. Being living organisms, the products are much more sensitive to water quality and especially traces of disinfectants. It is normally best to avoid temperatures above 30°C or below 10°C, drought-stressed plants, very sunny conditions (unless a UV protectant is included) and low-humidity conditions, all of which reduce drying time and increase the risk of phytotoxicity to the plant or destruction of the BCA.

Tank-mixing with conventional fungicides may also present some problems (particularly with emulsifiable concentrates) but many BCAs have been selected for insensitivity to common conventional fungicides so that both products remain active (Ojiambo and Scherm, 2006). This information is often disclosed on the label of the biological.

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As with conventional fungicides, the efficacy of some BCAs may be improved with adjuvants that improve coverage (spreaders) or adhesion (stickers). As always, it is sensible to carefully test a few plants prior to widespread application to confirm that the combination is not phytotoxic to the plant, nor does the adjuvant reduce the efficacy of the BCA. It is also necessary to carefully select the appropriate phenological state of the crop to obtain the best results. These factors differ between products and crops.

Unlike conventional fungicides, BCAs often have distinct storage conditions, based upon the product or microorganism used. Storage temperatures need to be well above freezing, and protected from excessive heat, but some require refrigeration. Failure to properly store BCAs under the appropriate conditions results in product degradation or a loss of viability and a resulting decline in efficacy (Ojiambo and Scherm, 2006). Furthermore, botanicals need to be used soon after the product is opened, and the product may not be effective later due to contamination, degradation or oxidation.

Copper formulations

Early studies testing the efficacy and persistence of different copper compounds found none compared favourably with Bordeaux mixture although phytotoxicity was an issue on many different species and genera of plants. In general, the basic copper sulfates were the least phytotoxic to plants but only moderately effective; the copper oxychlorides (COCS formulation) couple efficacy with minimal phytotoxicity when used appropriately.

Regardless of formulation, the bioactive component of copper fungicides are the cupric ions (Cu^{2+}) that are released into the spray solution. The inclusion of lime as a safener in fixed coppers reduces copper solubility and the release of copper ions in solution. However, the solubility of copper alone cannot explain the efficacy of the highly mobile copper sulfate, as compared with the slightly soluble copper hydroxide and copper oxychloride as well as the insoluble copper oxide. Copper fungitoxicity is a more complex mechanism than simply solubility and may explain the efficacy of less water-soluble copper compounds and differences observed between pathogens, formulations and efficacy. In one study, Montag *et al.* (2006) demonstrated that VENTIN spore exudates react with insoluble copper compounds to form more toxic copper complexes than the dissolved Cu^{2+} ions alone, similar to work done 50 years earlier by Arman and Wain (1958). Previous research identified that the concentration of Cu^{2+} ions on leaves is a dynamic equilibrium between the soluble and complexed forms of copper (Menkissoglu and Lindow, 1991). Not surprisingly, plant structural differences (e.g. cuticle thickness, stomatal and trichome density, etc.) impact the availability and the absorption of Cu^{2+} ions by leaf surfaces (Fu *et al.*, 2015). Regardless of formulation, copper efficacy is highly correlated to the particle size used in the formulation and how well it is adsorbed by the plant – there is little difference in the level of control per unit of metallic copper. In general, copper fungicides formulated with smaller particles provide efficacy by way of better adsorption, coverage, rainfastness and persistence. However, these small particles are usually less persistent than larger, less-soluble particles.

To be effective, copper needs to adsorb to the plant surface to form a protective coating that releases the copper ions. When water contacts the treated plant, insoluble fixed copper becomes solubilized, the degree of which depends upon the pH of the water, with lower-pH water (acidic) releasing more copper ions than high-pH water (alkaline). Copper compounds function as protective fungicides with good residual activity (e.g. Hamilton, 1931; Holb and Heijne, 2001). However, a recent *in vivo* study on VENTIN demonstrated that some copper salts ($Cu(OH)_2$ and $CuSO_4$) showed 16 and 40 h post-infection activity (Montag *et al.*, 2006). Post-infection activity of copper fungicides requires leaf wetness (because of high humidity or cooler temperatures) and increases the risk of phytotoxicity to the plant host.

The solubility of copper is inversely proportional to its persistence (Table 6.6), so care must be taken to balance the available amount of free copper to protect the plant versus too much copper that can injure the plant, or simply run off and accumulate in soil. Copper ions can be phytotoxic and can damage plant tissues, particularly with young growth that has not yet hardened off. Slow drying conditions also increase

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Table 6.6. Comparison of solubility and persistence of copper formulations at 20°C, pH 7.0. (Authors' own table.)

Copper formulation	Solubility (mg/l)	Persistence
Cuprous oxide	Insoluble	
Copper oxychloride	0.0000001	
Copper hydroxide	1.19	
Tribasic copper sulfate	3.42	
Copper sulfate	142	
Copper chloride	730	

copper ion availability. Therefore, longer wet weather periods with low temperatures after a spray application can be phytotoxic and may cause fruit russeting during bloom and early fruit development (e.g. Ellis *et al.*, 1998; Holb and Heijne, 2001).

The broad-spectrum biocidal ability of copper, like other broad-spectrum pesticides, means that it has significant environmental impacts, especially on soils and waters, and this runoff is a source of concern. Although it is an essential nutrient, excess copper is toxic to plants, beneficial microbes and animals (Beckerman, 2008). Excess copper interferes with decomposition, disrupting nutrient cycling and mineralization of the essential plant nutrients, nitrogen and phosphorus. In plants, soil accumulation of copper can cause a retardation of crop growth. Excess copper can accumulate in some higher animals, while other aquatic organisms (from insects to fish and birds) suffer more acute toxic effects. In humans, excess copper can result in heavy metal poisoning and have serious health effects. Long-term use of copper in agricultural production has resulted in soil copper levels that exceed the safe limits (Holmgren *et al.*, 1993; Paoletti *et al.*, 1998). Misapplication and misuse of copper fungicides (i.e. high rates and *ex post facto* applications) have contributed greatly to this problem. Excess copper in orchards has been shown to negatively impact soil ecology and invertebrate levels (Holmgren *et al.*, 1993; Lamichhane *et al.*, 2018).

Copper resistance

One significant advantage of most BCAs, botanicals and basic substances is that they have proved to be at low risk of resistance evolution. This was also thought to be the case for copper but evolved and innate resistance to copper has been observed in a number of pathogen species. Many bacteria and a few fungi are resistant to copper and have different mechanisms of resistance including, but not limited to: efflux by multi-drug resistance efflux pumps; ATPases; reduction in biochemical target sensitivity, oxidation, reduction, methylation and dealkylation; laccases; and extracellular chelation by secreted metabolites (Giller *et al.*, 1998; Lemire *et al.*, 2013) (Fig. 6.3).

Although widespread resistance is a problem with many bacterial plant pathogens, some fungi have demonstrated considerable tolerance to copper, namely yeasts and wood-decaying basidiomycetes. This type of 'tolerance' is distinct from resistance, and often a result of extracellular precipitation, complexation and crystallization, biosorption to cell walls, pigments and extracellular polysaccharides (Gadd, 1994).

Sulfur formulations

Sulfur dusts are composed of elemental sulfur formulated with inert materials – talc, bentonite, kaolinite, aluminium silicate ($Al_2Si_2O_5(OH)_4$), pyrophyllite (aluminium silicate hydroxide: $Al_2Si_4O_{10}(OH)_{20}$). Ground sulfur (or brimstone) can be used as a dust directly applied to plants. In the absence of micronizing (grinding to a fine particle size with a micronizing mill as opposed to other methods that pulverize the sulfur), ground sulfur can vary in size from 4 to 250 µm in diameter. The addition of inert materials minimizes clumping. Early sulfur fungicides were often formulated with arsenic and lead for added efficacy.

The wettable sulfur products are processed elemental sulfur combined with a colloidal material (usually proprietary) that serves as a wetting agent and creates a colloidal suspension of the sulfur. By itself, elemental sulfur is hydrophobic,

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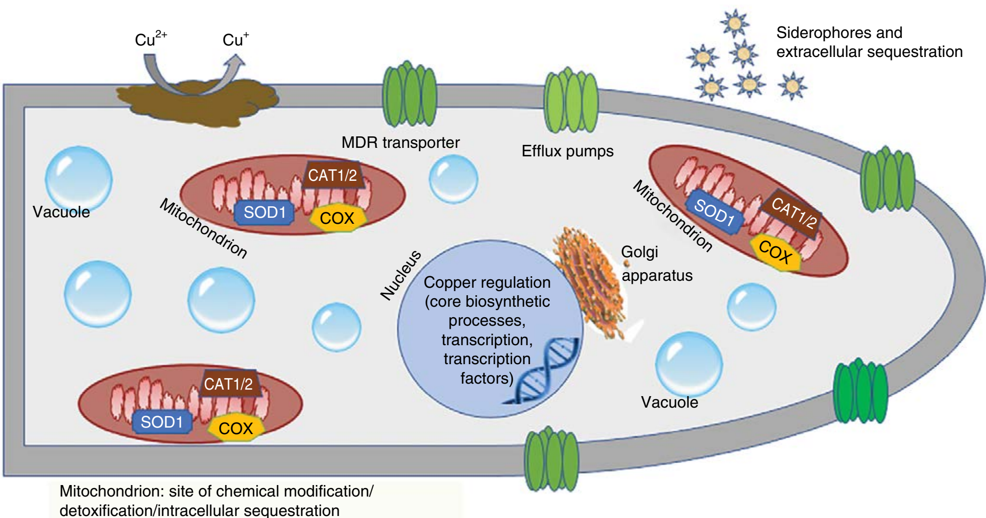

Fig. 6.3. Mechanisms of resistance to copper. MDR, multi-drug resistance; CAT, catalase; SOD, superoxide dismutase; COX, cytochrome oxidase. (Authors' own figure.)

so wetting agents (e.g. Triton B 1946, colloidal adjuvants, etc.) are necessary to create a fungicidal spray, along with a dispersant to prevent clumping or caking.

Commonly formulated as a mixture of 29% (w/v) calcium polysulfide and a small amount of calcium thiosulfate, which results from boiling hydrated lime ($CaO{\cdot}H_2O$) and elemental sulfur with water (McCallan, 1967), the lime sulfur formulation was essentially standardized by the 1850s, and was in common use for apple scab, powdery mildew, aphids, mites, brown rot and other pests and diseases (Tweedy, 1969). Early investigations found that its efficacy and phytotoxicity were similar to copper fungicides (Hamilton, 1931; Gadd, 1994) but that lime sulfur also provided post-infection control when applied within 30–50 h after inoculation of VENTIN (Mills, 1947). More recent investigations on lime sulfur efficacy in organic apple disease management found a greater curative effect of lime sulfur against apple scab in wet years, but that this efficacy came with significantly higher foliar phytotoxicity and fruit russeting compared with drier years (Holb and Heijne, 2001; Holb *et al.*, 2003). *In vivo* studies found that 1.5% (w/w) lime sulfur applied as late as 16 h after infection killed early infection structures and stopped further development of VENTIN (Montag *et al.*, 2006). The curative activity of lime sulfur requires continued leaf wetness after spraying to facilitate polysulfide release and penetration of the fungus (Trapman and Drechsler-Elias, 2000). Should the leaf surface dry, lime sulfur functions as a protectant fungicide against VENTIN and presumably other fungi (Tweedy, 1981; Trapman and Drechsler-Elias, 2000).

Disease Control in Organic Agriculture

'Organic' farming enterprises operate under a range of rule structures in different countries and states. Most Organic farming certifiers are independent non-governmental organizations, but many enjoy a good level of governmental support especially in the EU, UK and USA. Growers must subscribe to one of these Organic certifiers for the right to attach their logo to their products and hence attract a premium price. Likewise, the producers of biological fungicides must get these Organic certifiers to add their stamp of approval to their products.

From the outside, the rules of Organic farming, especially as they apply to crop protection, appear to be a series of prohibitions; synthetic pesticides are not allowed, and neither are synthetic fertilizers such as superphosphate and Haber–Bosch nitrogen sources. All means of

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genetic modification including genome editing are banned, although other methods of crop breeding are allowed. Instead, Organic farmers rely on a suite of agronomic practices that seek to promote healthy soils and healthy crops by the judicious use of crop rotation, resistant cultivars and soil amendments such as farmyard manures and legume crop residues. Although Organic farming businesses are linked by these similar rules and regulations, we should appreciate that they are every much as diverse as conventional farming operations. Some grow monocultures intensively using biological pesticides, relying on premium prices in niche markets to turn a profit. Others are mixed arable, horticultural and animal husbandry enterprises relying on product and landscape diversity to keep diseases in check while enjoying a multiplicity of direct and indirect income sources.

The diseases suffered by Organic horticultural and arable growers are no different from the ones afflicting conventional growers. All farmers rely of four methods of disease control: biosecurity, agronomy, genetics and chemistry. The reduced armoury of chemicals means that Organic growers are more restricted in their options. A key requirement for Organic growers is to select crop species and varieties with good levels of genetic disease resistance for all threatening diseases. As we have seen, Organic farmers make heavy use of BCAs, botanicals and basic substances. There is a greater need to employ agronomical practices that reduce disease pressures such as tillage, crop rotation and crop hygiene. It is also important to select seed lots that are free of seed-borne pathogens as opposed to using one's own saved seed. Early planting dates can often result in some disease avoidance as the crop can be lifted before the disease is rampant, for example to control PHYTIN in potatoes (Finckh *et al.*, 2006). It is also more important to avoid drought or waterlogging as these often make crops more vulnerable. Lower seeding rates and lower rates of N:P:K nutrition fertilizer are desirable. It is well established that overfeeding with nitrogen promotes powdery mildew disease, and that the addition of potassium can give protection (Solomon *et al.*, 2003).

There has been much debate about the use of cultivar mixtures and other forms of genetic heterogeneity to achieve sustainable disease control. This can be achieved by using mixed cultivars, each one with a different set of disease resistance alleles, or by intercropping, where a different species of plant is placed in between the crop. In both cases the theory is that the rate of spread of the pathogen would be lower than for a monoculture as the spores from a successful infection are less likely to alight on a susceptible host and propagate the disease. In general these methods increase disease control (Kristoffersen *et al.*, 2020). One downside of mixed cropping is that the harvested product will also be mixed and may therefore attract a lower price. However, this is not generally the case when the crop is destined for animal feed markets. Another downside of intercropping is that competition between the two species will reduce the yield of the more valuable crop compared with the monoculture. However, the second crop may have other useful attributes that compensate for the loss of gross yield.

One difficulty with crop heterogeneity methods, intercropping and mixed cropping, arises if disease control fails and it becomes necessary to apply an in-season crop protection method. It is usually impractical to spray just the affected cultivar or species, so it will be necessary to use the same volume of product per unit area as would be the case for a denser, uniform crop. This is unlikely to matter very much in the case of biofungicides. BCAs rely on growth of the applied organism on the crop and on spread from plant to plant. Just as heterogeneous crops delay the spread of a pathogen, it also delays the spread of a BCA.

Comparing crop protection in conventional and Organic cropping

It is very difficult to do meaningful experiments that directly compare conventional and Organic cropping. Organic certification requires multiple years of adjustment, meaning that many factors would differ between an Organic and a neighbouring farm that could explain any difference in disease control. Many countries host research farms devoted to Organic farming studies and much of the research emanates from these centres (van Bruggen *et al.*, 2016).

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Nonetheless there are numerous publications, published mainly by specialized Organic farming researchers, comparing disease outcomes in somewhat comparable situations. While there are some cases of increased disease in Organic production, the commonest published result is no difference or a disease reduction. Among the latter are cases of reduced mycotoxin contamination in Organic growing (Birzele *et al.*, 2002) although a more recent review suggests that results are inconsistent (Magkos *et al.*, 2006). The explanation is that a conventional fungicide is too efficient at killing fungal spores leaving the developing cereal head vulnerable to infection at a later date. Another case is the reduction in cereal powdery mildew in Organic versus conventional farms, despite the application of fungicides in the latter (Daamen *et al.*, 1989). This is attributable to the observation that powdery mildews are promoted when nitrogen fertilizer is in excess (Solomon *et al.*, 2003) and can be combatted by added macronutrients (Brennan and Jayasena, 2007). Overall any decreased disease in the Organic field is likely outweighed by the higher yield in the conventional (Schrama *et al.*, 2018). The economic basis of Organic farming thus depends on a higher product price.

Even though comparisons of Organic and conventional farming rarely generate clear-cut and statistically significant differences on the efficacy of different disease control procedures, there is much to be learnt and many experimental leads to follow up. Many disease pressures in conventional farming are due to pushing the system too hard with high sowing densities, fertilizer rates and short rotations. A relaxation of these pressures can lead to more profit as the reduction in input costs can outweigh the reduction (if any) in yield (Jorgensen *et al.*, 2017). Ultimately the success or failure of an Organic cropping programme will depend on the balance of inputs costs (including certification) versus income from crop sales, subsidies and ancillary income streams.

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7

Formulation

Key Points

- Fungicide AIs must be mixed with other materials to be packaged, transported, sold and delivered – the mixture is called the formulation.
- Formulations optimize AIs for a diversity of uses including foliar applications, seed treatments and postharvest protection.
- Formulation includes adjuvants and surfactants to improve efficacy, facilitate application and ensure safety to the applicator and the crop.
- Nanotechnology is a formulation technology that has the potential to improve fungicide delivery and plant protectants by reducing application rates, providing controlled release, but with increased efficacy of the AI.

Introduction

Multiple challenges exist in delivering a biologically active molecule to prevent or interrupt the infection process of the target pathogen. The AI – *active ingredient* – of a fungicide product must be formulated to work on a diversity of crops and against a great diversity of pathogens. The mixture of the AI(s) and other necessary materials is called the *formulation*. Formulations are vehicles which enable the active material to be applied to the crop under a variety of conditions without loss in performance. They should be:

- safe to the crop and applicator;
- easy to handle and straightforward to apply;
- compatible with other major products;
- acceptable to registration authorities; and
- suitable for large-scale manufacture.

Formulations consist of the AI and inert ingredients like carriers, solvents, adjuvants, surfactants and stabilizing agents that enable the active material to be applied to a diversity of crops under a variety of conditions without loss in performance. Logically, the formulation of fungicides should match the complexity of the many interacting factors that affect their performance in controlling disease. These include the host plant, the pathogen, the target stages of fungal development, the biochemical target and the delivery system. However, the fungicidal activity of compounds submitted for laboratory and glasshouse screening tests is usually determined using simple formulations, for example aqueous acetone solutions, and such rudimentary systems may favour those characteristics. Laboratory formulations used in screening are not suitable for use in commercial situations and further work is required to present the AI in a practical form.

The choice of formulation, including adjuvants, surfactants and solvents, can promote or inhibit the uptake of fungicides (Stock, 1996) to

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the extent the physicochemical properties of the AI allows. Formulation strategies have to be designed for each new active material. Preventing losses through volatility will disadvantage a product that is redistributed in the crop through the vapour phase. Similarly, formulation components that promote retention and prevent wash-off by improving fungicide uptake may remove the ai from the site of disease control. The strobilurin kresoxim-methyl is unstable in plants and so is an example of a product that must be formulated to minimize penetration (Köhle *et al.*, 1994). In other cases, safeners, such as lime, are necessary to prevent phytotoxicity, such as in the case of copper and sulfur fungicides (Richardson, 1997).

Formulations are essential to ensuring that the biologically active part of the fungicide is consistently delivered to the appropriate plant organ (e.g. seed, foliage, fruit, root) at the appropriate concentration to control the pathogen but not harm the plant. Successful fungicide formulation improves the physiochemical properties of the AI. These properties include solubility, lipophilicity, particle size, pH and the acid dissociation constant (pK_a) of the AI. In some cases, inventive formulation may enhance redistribution, mobility and overall performance, as in the case of the microencapsulation of surface-acting fungicides which serves to reduce losses through volatile action while increasing the persistence of the product and hence lengthening the period of acceptable control.

Properties of lipophilicity and solubility are the primary barriers to plant uptake of fungicides. For a fungicide to successfully protect a plant, it must be *adsorbed*, in the case of contact/protectant fungicides, or be *absorbed* by the plant to work systemically. This is a primary distinction in fungicides: contact, protectant fungicides are adsorbed to the surface of the plant, whereas penetrant/systemic fungicides are absorbed by the plant. Upon absorption, the AI may be *locally systemic*, being distributed only nanometres from the application site, to slightly more systemic and travelling micrometres from the droplet site. Other fungicides may be *xylem-mobile*, moving upwards through the plant, or *amphimobile* and be distributed via the xylem and the phloem throughout the plant (for more details see 'Penetrant Fungicides' section in Chapter 8, this volume).

Formulation improves fungicide efficacy in many ways, from improving droplet coverage, adhesion and retention to the target; to facilitating or preventing adsorption and absorption; and to increasing efficacy and safety to the user and the crop. Although the primary route of pesticide delivery occurs via spraying, other means of pesticide application include seed treatment, injection and slow-release soil-borne granules (see 'Formulation Types' section below), all of which require different formulations and doses. All formulations have advantages and disadvantages: the trade-offs include cost, user need, site of action, type of equipment delivering product, timing and dose. The type of formulation ultimately chosen is dependent on all these factors.

Formulation Types

Fungicides are formulated to improve their efficacy, facilitate ease of application and improve their safety to the applicator and the crop; the type of formulation employed depends on the physical characteristics of the AI and the needs of the market. Formulations can be solid (i.e. mixtures of dry ingredients) or liquid (solutions, emulsions or suspensions), and many fungicide AIs have multiple types of formulation to address different needs (e.g. root versus foliar protection), different stages (i.e. early protection of seedlings or cuttings versus mature plants) or different crops (with different pesticide tolerances) (Table 7.1). Dry formulations are usually less expensive to manufacture, are more stable when stored properly and kept dry, and are more amenable to bulk transportation. Unfortunately, dry formulations are often abrasive and damage nozzles, and are prone to line blockage. Liquid formulations usually contain a higher concentration of AI, are easy to mix and keep in solution, and eliminate the risk of dust inhalation, but transport may be an issue due to the use of solvents.

Dust (D) formulations are manufactured by grinding the fungicide, together with a solid diluent, in a ball mill. Particle size is maintained at about 20 µm diameter. The size is a controlled

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Table 7.1. Common pesticides formulations, composition and uses. (Authors' own table.)

Formulation	Composition	Pros	Cons
Solid (dry)			
Dusts (D, DP)	Consists of active ingredient (AI) and dry inert carrier that may be ash, chalk, clay, talc, etc.	Ready to use; low concentration of AI	Low concentration of AI; can drift; residue easily removed; inhalation risks
Granules (G)	Composed of carrier containing the AI; most commonly used to prevent root and crown rots; best used when mixed with soil or soilless mix to allow for slow release of fungicides to protect roots as particles break down	Slow release; pourable	May require special equipment (mixers or spreaders) for application or incorporation into the soil or soilless media; dust
Water-dispersible granules (WDG, WG)	Larger particles of active and inert ingredients better protect applicator from dust; often require continuous agitation to maintain suspension	Can be easily poured; less dust; rapid dispersion	Dust can still be a problem; dispersing agents generally prevent precipitation, but agitation may be required to keep product in solution
Wettable powders (WP)	Finely ground particles consisting of both active and inert ingredients that do not contain organic solvents	Simplest formulation and often the most economical; similar to flowables when added to spray tank	May require scooping out of bag; dusts may pose danger to applicator; require continued agitation to maintain in the spray solution
Water-soluble packet (WSP)	Pre-measured and packaged WP in water-soluble pouch	Facilitates use; protects applicator from dust	Cost; WSPs need to dissolve fully before other tank-mix partners are added to ensure packet dissolution and prevent blockages; generally reserved to larger volumes of fungicides
Liquids			
Emulsifiable concentrate (EC)	Organic solvents (oils) are used to dissolve the less-soluble, lipophilic fungicides, and emulsifiers permit the suspension of an aqueous liquid in an oil; the emulsifier exists at the aqueous–oil interface, reduces surface tension and stabilizes the two liquids	Allows for more concentrated delivery of fungicide	More phytotoxic than other formulations; can carry other pesticides or plant growth regulators into the plant; can 'cream out' when larger molecules coalesce and separate from the emulsion

Continued

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Table 7.1. Continued.

Formulation	Composition	Pros	Cons
Flowable (F)	Finely ground formulations (at the micro- to nanoscale) with added suspension agents to keep particles in solution	Fungicide disperses rapidly in the water; inclusion of wetting agents required for most lipophilic fungicides; have the potential to improve application	The solids can settle out as the wetting agent is diluted in the spray tank; foaming can result, along with aggregates that clog filters
Microemulsion (ME), microemulsion concentrate (MEC)	An EC, but the emulsion forms at the micro- or nanoscale	Ease of mixing; agitation may not be needed; oil will not separate out	MEs contain a lower concentration of AI than MECs; MECs are often formulated with higher concentrations of surfactants and are more phytotoxic
Suspension concentrate (SC)	Similar to F, but combining the properties of an EC and WP	Easy to handle and apply; does not contain solvents; less phytotoxicity	As with powders, abrasion may be an issue; poor storage stability
Suspension emulsion (SE)	Aqueous formulation of suspended solids (SC) and emulsified droplets (EC)	Rapid dispersion in water	Require agitation after dilution to maintain dispersion; prone to heterogeneous flocculation, with product precipitation and screen blockage

balance between the avoidance of particle coagulation (diameter too small) and an unacceptable reduction in activity (diameter too large). Dusts are difficult to use and tend to be the least effective of fungicide formulations because of losses during application due to drift (losses due to wind).

Wettable powders (WP) are solid formulations suitable for compounds that have low aqueous solubility. They, like dusts, are produced by crushing a mixture of the active and a solid, inorganic diluent such as clay in a ball mill to a particle size of <25 µm. Wetting agents and dispersion agents are added to assist in particle suspension during application. Other adjuvants may be included to improve persistence (stickers) and photolytic stability (UV filters). Wettable powders are by their nature dusty and are potentially hazardous to handle. Despite these difficulties, many immobile fungicides are formulated as wettable powders.

Granular fungicides (G) are applied dry and are intended for soil applications to protect the roots and crown of plants. Granule formulations are easy to apply, are stable in high wind and being relatively heavy, have good crop penetration characteristics. At its simplest, a granular formulation consists of the inert carrier and the fungicide, or even a liquid fungicide sprayed on the granule for later distribution. Most formulations, however, are more complex. The weight and structure of granules provide mass to carry them through foliage to the ground below, structure for soil integration and may provide a slow release (depending upon formulation) of fungicide for extended protection. Granule formulations are produced by adsorption of fungicide on to the surface of porous substrate pellets that range in size from 0.5 to 1.5 mm in diameter and must pass through a specifically sized sieve (in this case, a 4-mesh, meaning four square openings per square inch; 25-mesh means 25 openings per square inch, etc.) and be retained on an 80-mesh sieve.

When a fungicide is formulated as granular, carriers are added to create a solid structure.

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These carriers can be divided into inorganic (mineral) and organic. Mineral carriers include attapulgite/palygorskite, bentonite, kaolin, montmorillonite and other clays for more controlled-release applications, diatomaceous earth (more often used for insecticides), gypsum, limestone, and sand, to name but a few. Organic carriers include inexpensive waste products, such as maize cobs, nut shells, groundnut hulls, recycled paper fibre, starch, and even pasta to support some biologicals. More expensive products, like acrylic and other polymers, alginate or polyisonitriles, and nanotechnologies can be used for controlled-release formulations. The benefits of granules include the reduction in drift potential and inhalation, but their bulk does present problems, particularly for producing uniform applications. Lastly, granular formulations, when not properly incorporated or broadcasted, can be ingested by birds and other animals.

Suspension concentrates (SC) are formulations formed from fungicides that have been ground to a fine powder (<5 μm), suspended in either water or an organic liquid and then blended with a solid inert carrier plus suitable adjuvants to suspend the AI. As in wettable powders and dusts, particle size is critical to the performance of the fungicide: too large a particle size may reduce performance. In addition, the choice of adjuvant profoundly affects the utility of the formulation. Suspension concentrates with wetter often give corresponding activity to emulsifiable concentrates. Without wetter, the performance may be reduced or, in extreme cases, removed. Such effects can frequently be related to a lower level of penetration into the leaf by the fungicide. Fungicide phytotoxicity, usually most apparent in emulsifiable concentrates, may be reduced to an acceptable level without loss in performance by formulation as a suspension concentrate with the addition of the appropriate type and amount of adjuvant.

A modification of the suspension concentrate is microencapsulation. Here the fungicide is incorporated into a small, polymer-based sphere (≥15 μm diameter) which is permeable to enable the controlled release of the active material. They are available as microencapsulated flowable concentrates comprising the capsules and suitable wetting agents. Unlike wettable powders, suspension concentrates do not present dust hazards. They can be easily dispensed and are more convenient to use.

Adjuvants

Adjuvants are ‘materials that are added to a tank mix to aid or modify the action of an agrichemical, or physical characteristics of the mixture’ (ASTM, 1999; Hazen, 2000). The addition of adjuvants can profoundly affect the spread, retention, penetration and spray efficacy of materials being applied to plant surfaces, and when combined with fungicides, may potentially improve disease control by increasing the fungicide’s penetration, improving the fungicide’s dispersion (spread) or extending the fungicide’s persistence on the plant. For these reasons, they are routinely screened in combination with new materials in the laboratory: for example, small amounts of some alcohol ethoxylate surfactants benefit the curative activity of dimethomorph (Grayson *et al.*, 1996); adjuvants may increase the initial penetrative properties of fluquinconazole, thus enhancing redistribution and hence performance (Stock, 1996). The addition of Synperonic A5, a lipophilic alcohol ethoxylate, to prochloraz promotes the foliar penetration of the fungicide to a point that effectively removes most of the applied product from the leaf surface (Stock, 1996) (Fig. 7.1). Adjuvants may also be added to tank-mixes by applicators to improve the performance of a product in the field (Percich and Nickelson, 1982; Steurbaut, 1993; Gent *et al.*, 2003; Gaskin *et al.*, 2004; Abbott and Beckerman, 2018). Formulation may even be modified to inhibit fungicide action, as in the removal of activity of prochloraz in wettable powder formulations. For these reasons, adjuvants require registration (Chapman and Mason, 1993).

Adjuvants include acidifiers, anti-flocculants, buffers, crop oils, defoamers, emulsifiers, fertilizers, penetrants and surfactants, to name but a few. It is important to note that many recently released pesticides are pre-packaged with adjuvants (called additives), which are listed as ‘inert ingredients’ on product labels. The propriety nature of many companies’ inert ingredients reflects why certain pesticide formulations are more effective than others in controlling certain pests, despite equal amounts of the same AI. As a result, fungicides are formulated in several ways, depending on their physical characteristics and on the needs of the market. The addition of adjuvants can profoundly affect the performance

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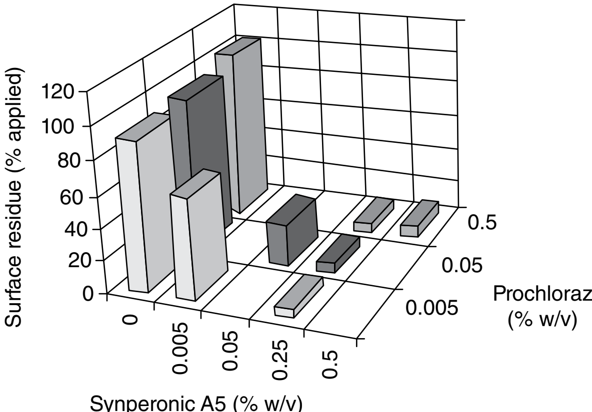

Fig. 7.1. Effect of Synperonic A5 on uptake of prochloraz into wheat leaves. (From Stock, 1996.)

of fungicides and they are routinely screened in combination with new materials.

Surfactants

Most commercial fungicides are relatively insoluble in water, preventing uptake, translocation and movement to the sites of action. To circumvent this, fungicides are often dissolved in lipophilic, non-polar, organic solvents such as xylene or cyclohexane. These solvents are insoluble in water and mixtures of the two separate rapidly into layers. A fungicide dissolved in the lipophilic solvent would fail to dissolve into the aqueous fraction and would not be delivered during part of the application process. To address this, surface-active agents (surfactants) and/or emulsifiers are added to the organic solvent–fungicide mixture.

Surfactants can be anionic, cationic or non-ionic. *Non-ionic surfactants* (e.g. polyethylene ethers) do not have a charge, are compatible with most pesticides and improve fungicide coverage on the waxy surfaces of target crops by reducing surface tension. Such spreaders have a greater solubility in organics than ionic surfactants. *Anionic surfactants* are negatively charged and used with salts or acids. As such, they are rarely used with the non-polar fungicides, but can be used as dispersants or compatibility/emulsifying agents. *Cationic surfactants* are used the least, due to issues of phytotoxicity. There is one important exception to this: dodine (dodecylguanidinium acetate), a fungicide used in fruit production, is itself a cationic surfactant. Most formulations contain a mixture of non-polar and anionic emulsifiers. Some fungicides have inherent surfactant (cationic) properties, like dodine, and in these cases the addition of anionic surfactants is avoided.

The *organosilicone-based surfactants* consist of a hydrophilic head moiety and a hydrophobic tail. These products are often non-ionic, and include polyglycerol alkyl ethers, ester-linked surfactants, polyoxyethylene alkyl ethers, sorbitan esters and polysorbates. Characteristics of this group include improved rainfastness, low surface tension and, depending on the formulation, stomatal infiltration and even the ability to form *niosomes*, a self-assembling, non-ionic vesicle of 10–100 nm, for nano-delivery of pesticides (Fig. 7.2). Gaskin *et al.* (2002) highlighted the importance of matching organosilicone adjuvant concentration with application spray volume to improve spray distribution and retention.

Some surfactants (called *spreaders*) are wetting agents that reduce the amount of beading (surface tension) of the droplet on the leaf surface, thereby improving fungicide coverage (Figs 7.3 and 7.4). Surfactants improve fungicide performance by bringing the AI into extended and closer contact with the leaf surface, thereby improving the potential of the plant to adsorb or absorb the fungicide. Spreaders generally lower the surface tension of the spray solution to 30–50 dynes/cm and may increase capture of the target.

Surfactants that help fungicides adhere or 'stick' to plant surfaces are called *stickers*. A sticker

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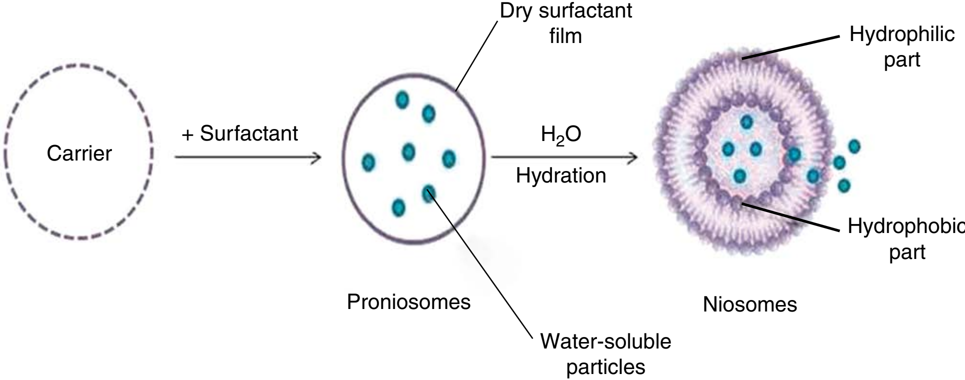

Fig. 7.2. Niosomes formation from provesicular forms by hydration. (From Yasam *et al.*, 2014, with permission.)

improves how the fungicide adsorbs to the leaf surface, thereby improving persistence (stick) or adhesion. Stickers also can decrease the rate at which rain can wash the fungicide off or sunlight can break the fungicide down (photodegradation). These processes can be combined into adjuvants referred to as *spreader-stickers*.

Acidifiers are surfactants that lower the pH (acidify) of a spray-tank solution. This is particularly important for a fungicide like captan, which has a half-life of 20 min if the water has a pH greater than 8.0 (alkaline). In other words, captan breaks down and is considered ineffective in 40 min under high-pH conditions. An acidifier will improve captan performance if the tank water has a pH of 8 or greater (Wolfe *et al.*, 1976). However, with a fungicide like copper, an acidifier could make the copper phytotoxic, which could damage the plant (particularly flowers and fruit) or even kill it. This is but one example of the care applicators must take to choose the right adjuvant–pesticide combination. To ensure the maximum effectiveness of pesticide applications, the applicator should measure the pH of the water with the tank-mixes in the spray tank, and add the appropriate buffering agents, if necessary, to adjust the pH to neutral (7.0). There are many commercial buffering agents available.

Penetrants, as the name suggests, allow penetration of the fungicide into the plant. Penetrants are adjuvants that increase the ability of a pesticide to absorb and permeate into a living organism. Researchers have found that some penetrants improve disease management whereas other fungicide–surfactant combinations result in inconsistent to poor results with other penetrants and even phytotoxicity (see Abbott and Beckerman, 2018 and references therein). Such modifications may be advantageous or disadvantageous depending upon the proposed treatment timing and the growth pattern of the target pathogen. For example, it has been shown that small amounts of some alcohol ethoxylate surfactants benefit the curative activity of dimethomorph (Grayson *et al.*, 1996). Similarly, adjuvants may increase the initial penetrative properties of fluquinconazole, thus enhancing redistribution and hence performance (Stock, 1996). As previously mentioned, the addition of Synperonic A5, a lipophilic alcohol ethoxylate, to prochloraz promotes the foliar penetration of the fungicide to a point that effectively removes most of the applied product from the leaf surface (Fig. 7.1). While it might seem like a good idea to get a fungicide to penetrate into a plant, the reality is that many older fungicides like captan, chlorothalonil or copper are extremely toxic if they get into the plant, resulting in plant damage (phytotoxicity) and even widespread crop loss (Abbott and Beckerman, 2018). Because of the complex role adjuvants play in fungicide activity, many labels state that applicators must take care when using certain adjuvants with some fungicides to prevent phytotoxicity or chemical damage that can result in fruit russeting, plant injury and even plant death (Fig. 7.5). Lastly, combining adjuvants with many fungicides has the potential to improve disease management by reducing

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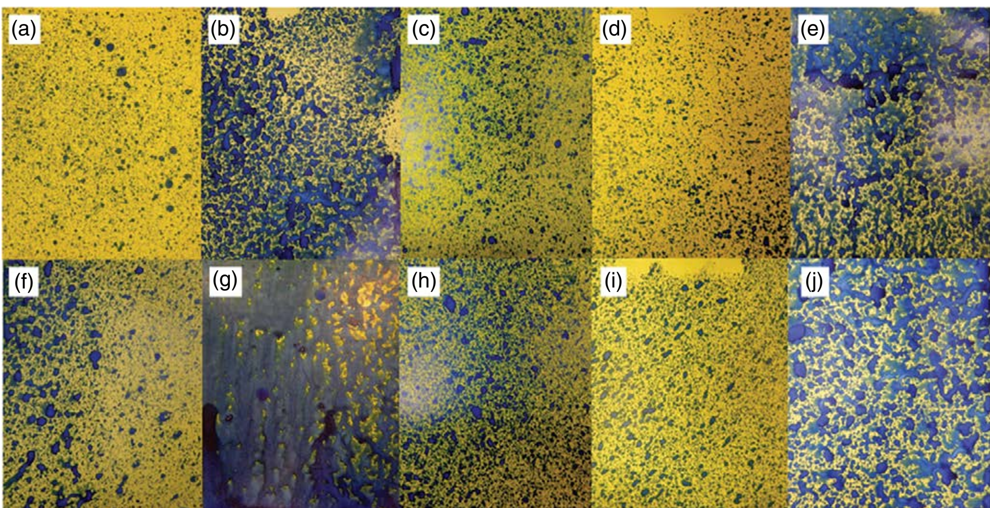

Fig. 7.3. The addition of different surfactants changed the surface tension of the spray solution, improving coverage of the water-sensitive paper and, more importantly, apples. The blue coloration indicates water coming into contact with the card. Treatments are: (a) control (H_2O) = 13% coverage; (b) Li700 = 68% coverage; (c) Bond Max = 27% coverage; (d) Attach = 16% coverage; (e) Latron B-1956 = 74% coverage; (f) captan = 27% coverage; (g) Li700 + captan = 92% coverage; (h) Bond Max + captan = 47% coverage; (i) Attach + captan = 27% coverage; (j) Latron B-1956 + captan = 71% coverage. (From Abbott and Beckerman, 2018.)

fungicide rates and extending the interval between applications. This can reduce pesticide use and, ultimately, increase a grower's net return in production. As always, the grower should carefully evaluate adjuvant–fungicide combinations in small test plots and be aware that different crops and even different varieties of a crop can respond in different ways.

Emulsifiers

Emulsifiers enable the suspension of otherwise immiscible liquid compounds to persist as suspended mixtures and prevent a return to immiscibility. Emulsifiers usually consist of a hydrophilic 'head' and hydrophobic/non-polar tail residues that interface between the liquids, preventing them from re-aggregating with other 'like' residues and leaving the emulsion (Fig. 7.6).

Commercial fungicides are generally not phloem-mobile and are relatively insoluble in water, being more soluble in lipophilic, organic solvents such as xylene or cyclohexane. It may be that the barriers to uptake, translocation and movement to the sites of action restrict what is possible in terms of physicochemical properties. Lipophilic solvents commonly used in formulations are insoluble in water and mixtures of the two separate rapidly into layers. A fungicide dissolved in the lipophilic solvent would under these conditions be largely absent in the aqueous fraction and, in the spray tank, would not be delivered during part of the application process. The addition of surface-active agents (surfactants), or emulsifiers, to the organic solvent–fungicide solution enables the formation of an emulsion comprising small spheres (<10 μm diameter) of organic solvent–fungicide in the sprayer. This type of formulation is the emulsifiable concentrate. Emulsions of fungicides formulated as emulsifiable concentrates should remain stable in the spray tank for at least 24 h to facilitate delivery. Because of their toxicity and fire hazard, organic solvents are being replaced by alternatives (e.g. microemulsions); where the active fungicide is soluble in water, the material may be formulated as a water-miscible liquid.

Emulsifying agents can be anionic, cationic or non-ionic. Non-ionic agents, for example polyethylene ethers, improve fungicide coverage on waxy surfaces of target crops by reducing

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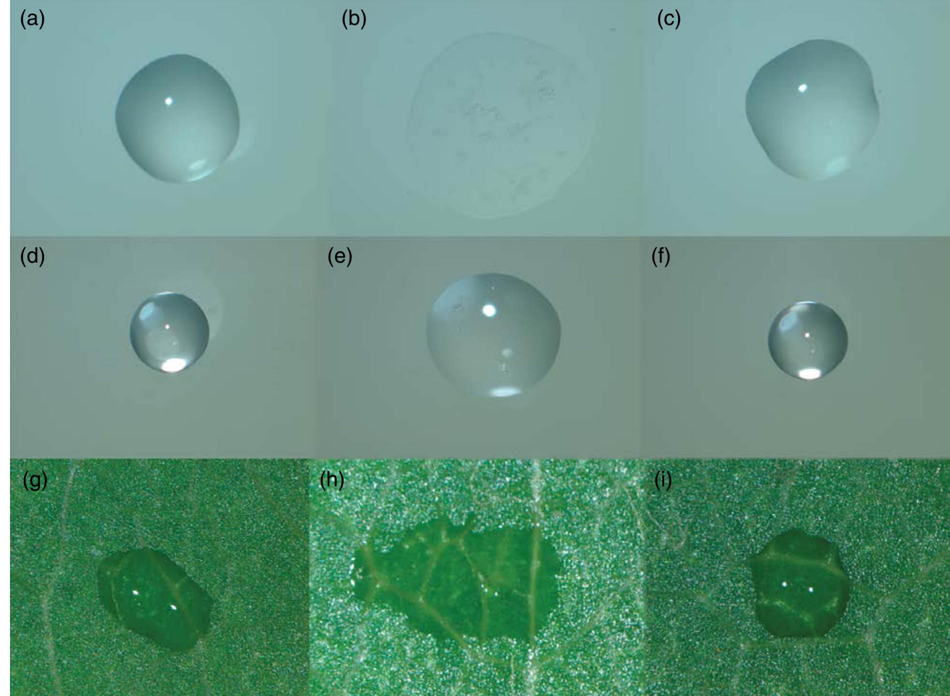

Fig. 7.4. Deposition patterns of a 343 μm droplet on hydrophilic, hydrophobic and crab apple leaf surfaces with three different mixtures containing an insecticide, a surfactant and/or drift retardant at 60% relative, respectively. All mixtures were formulated with distilled water as the carrier. (a) Hydrophilic surface, insecticide #2, A_0 = 0.642 mm^2; (b) hydrophilic surface, insecticide #2 + surfactant, A_0 = 1.215 mm^2; (c) hydrophilic surface, insecticide #2 + drift retardant, A_0 = 0.681 mm^2; (d) hydrophobic surface, insecticide #2, A_0 = 0.283 mm^2; (e) hydrophobic surface, insecticide #2 + surfactant, A_0 = 0.831 mm^2; (f) hydrophobic surface, insecticide #2 + drift retardant, A_0 = 0.301 mm^2; (g) crab apple leaf surface, insecticide #2, A_0 = 0.495 mm^2; (h) crab apple leaf surface, insecticide #2 + surfactant, A_0 = 1.103 mm^2; (i) crab apple leaf surface, insecticide #2 + drift retardant, A_0 = 0.499 mm^2. A_0 is the maximal coverage area of a droplet on the target surface after deposition. (From Yu *et al*., 2009, with permission.)

surface tension. Such spreaders have a greater solubility in organics than ionic surfactants and are favoured components of formulations where high water salinity in the spray solution can cause incompatibility problems with polar compounds. However, most formulations contain a mixture of non-polar and anionic emulsifiers. Some fungicides have inherent surfactant (cationic) properties and in these cases the addition of anionic surfactants is avoided.

Nanotechnology

Nanoparticles, due to their small size and high efficiency, have the potential to improve fungicide delivery and plant protectants by reducing application rates, providing controlled release, but with increased efficacy of the AI. The incorporation of older fungicides with a nanotechnology-delivery mechanism would be expected to reduce the dose of the fungicide and improve and extend efficacy over time. This is accomplished by manipulating AI particle size to the nanoscale. Most fungicides are ground via air mill to a 2–10 μm particle size (Backman, 1978) – 100 times larger than the proposed nanoparticle. The efficacy of a copper fungicide can be considerably improved by reducing the particle size (Horsfall *et al*., 1937) (Fig. 7.7). Kudsk *et al*. (1991) found that the rainfastness of mancozeb was inversely related to particle size and partly explained the differences observed between the formulations. Backman *et al*.

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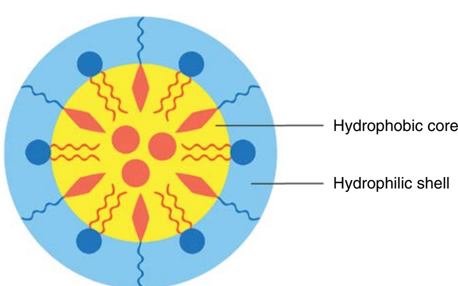
Fig. 7.5. Phytotoxicity damage to leaves due to inappropriate choice of adjuvant–pesticide combination. (Photo by Janna Beckerman.)



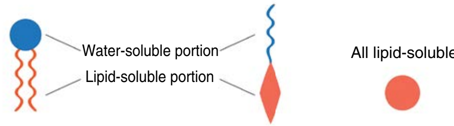


Fig. 7.6. When a surface-active emulsifier is combined with oil and water, a micelle is formed. Amphipathic molecules arrange themselves so that the polar (water-soluble) region is attracted to the water while the non-polar (lipid-soluble) region is attracted to the lipid fraction, allowing the components to combine.

(1976) also found that reduction in milling size provided better efficacy with less product.

Today, chlorothalonil milling size is currently at 2–5 µm. Assuming similar shape to the 20–50 nm proposed nanoparticle, the surface: volume ratio will increase by a factor of 10,000. Reduction in particle size and improvement of disease control occurs as a consequence of: (i) decreasing particle size increases the number of particles per gram, providing greater antifungal or antibacterial activity; (ii) as the particle size decreases there is concomitant increase in particle surface area that releases more AI when moisture is present; and (iii) smaller particles persist better as they are lighter and have a larger surface area relative to their weight (hence a greater area of contact with the plant surface), thereby resisting dislodgment, increasing adhesion and improving retention.

There is also a growing body of evidence that it is the surface area and not particle size that is the defining metric that controls toxicological interaction and renders nanoparticles more biologically active than their conventional counterparts (Oberdörster *et al.*, 2005). Although most pesticides are well adapted for application as emulsions, most fungicides are not, even with micrometre milling size, as the compounds are not sufficiently soluble in organic solvents to produce emulsifiable concentrates containing economic levels of AI. Nanotechnology would allow the rapid dissolution of hydrophobic fungicides, providing economic levels of AIs at greatly reduced rates.

Formulation of Biologicals

Biological fungicides (referred to henceforth as biologicals) require added considerations when formulating to maintain product efficacy, improve storage and improve application. This assumes the biological is amenable to mass production for commercial development; many biologicals that are found to be effective *in vivo* cannot be scaled up. Those that pass through this bottleneck require formulation to improve efficacy, extend shelf life and storage, and aid in delivery. Formulations for biologicals are similar to conventional fungicides and include dusts, granules, liquids, wettable powders and even microencapsulation (Schisler *et al.*, 2004). Some biologicals, such as the bacterium *Bacillus* and

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the fungi *Trichoderma*, readily lend themselves to formulation, due the ability of both to create spores. Spore 'durability' provides formulation scientists the opportunity to use processes that may otherwise be destructive (chemically, environmentally or physically) to microbes that do not have a resistant persistence structure.

As with conventional fungicides, adjuvants for biologicals include inert ingredients that improve the chemical and physical properties of the product but may also include nutrients for microbial growth. Obviously, many solvents, surfactants and fillers used for conventional pesticides cannot be used for biologicals (Table 7.2). Unfortunately, most details regarding the scaling up (fermentation) and formulation of biologicals are lacking due to their proprietary nature.

Formulation is an often overlooked yet essential component of fungicide development. Thoughtful formulation mitigates the realities that growers are faced with, including challenging environmental conditions, incomplete spray coverage and imperfectly timed applications. Formulations serve to improve application while reducing environmental impacts and are important factors in successful fungal disease management.

Fig. 7.7. Surface area affects all aspects of pesticide performance and behaviour. For example, a sugar cube (left), 1 cm in size, provides a surface area of 6 cm^2. Contrast this to sugar, caster sugar or powdered sugar (right) and how the surface area increases by these different millings of sugar. A nanopowder of sugar, 10 nm in particle size, would have a surface area 600 m^2, sufficient to cover a basketball court and most of the side lines. (Authors' own photo.)

Table 7.2. Inert ingredients play many different roles and are composed of a diversity of compounds. (Authors' own table.)

Inert product role	Example ingredients
Binders	Gum arabic, microcrystalline cellulose, carboxymethylcellulose, bitum, lime, gypsum, casein
Desiccants	Silica gel, anhydrous salts
Dispersants	Microcrystalline cellulose, lecithin
Inorganic carriers	Bentonite clay, kaolinite clay, diatomaceous earth, talc
Light blockers and UV protectants	HALS (hindered amine light stabilizers), lignin (PC 1307), oxybenzone
Nutrients	Molasses, peptone, casein, sucrose
Optical brighteners and dye markers	Blankophor BBH, Milori blue
Organic carriers	Flours, wood powder
Stabilizers	Lactose, sodium benzoate
Stickers	Pregelatinized maize flour, latex, pinene polymers
Surfactants	Tween (20, 80, 100), ionic and non-ionic surfactants, alkoxylated trisilanes
Thickeners	Gums, starches, xanthan gum

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8

Fungicide Mobility

Key Points

- Multiple terms are applied to how a fungicide moves in a plant and is a function of sorption.
- Contact fungicides are adsorbed.
- Systemic fungicides are absorbed.
- Fungicide uptake, redistribution and mobility are a complex interaction of the physicochemical properties of the AI, the formulation of the fungicide, environmental conditions and the target plant cuticle.
- Fungicide uptake and redistribution is predicated by the lipophilicity, particle size, pH and the acid–base dissociation constant (pK_a) of the AI.

Introduction

How a fungicide 'moves' in a plant has been described in many ways to clarify how these products work (Neumann and Jacob, 1987). Unfortunately, confusion rather than clarity has resulted. For our purposes, *mobility* describes fungicide movement after it is applied to a plant (as opposed to *systemicity*, which describes the ability of a fungicide to provide plant protection in areas not directly receiving an application or deposit). As such, what we are really describing is *redistribution*. Redistribution is a function of *mass transfer* (also called *mass flow*), which is literally the transfer of mass from one location to another, and describes how fungicides can give protection when not directly applied to the site (Klittich, 2014). For fungicides, the redistribution occurs via *diffusion* and/or *osmosis*. Diffusion describes the process by which molecules intermingle in a random fashion; osmosis is when molecules or atoms move from high concentration to lower concentration to achieve equilibrium. Diffusion occurs through both the cuticle and the plant cell membrane, and also the fungal cell wall and membrane if it is to be effective. For fungicide applications like soil drenches, osmosis is one mechanism for the adsorption of soil water and fungicides, and for the redistribution of some fungicides to the leaves of the plant.

The ability of an AI to redistribute is contingent upon its sorption. *Sorption* is the chemical and physical process whereby a compound becomes adsorbed or absorbed. Fungicides that adhere in an extremely thin layer to plant surfaces are *adsorbed*. Fungicides that can be taken up by the plant, and the AI dissolved or diffused, are *absorbed*. Because fungicides are either adsorbed or absorbed, they have two basic forms of mobility: contact (adsorbed to the surface) and penetrant (absorbed by the plant). Regardless of the type of mobility that a fungicide possesses, no fungicide is effective after the development of visible disease symptoms. For that reason, timely fungicide application before establishment of the disease is important for optimal disease management, followed by thorough coverage.

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Contact Fungicides

Contact fungicides are adsorbed. They are susceptible to being washed away by rain or irrigation, and most (but not all) do not protect new plant growth that occurs after the product was applied. Most of the older, multi-site fungicides (such as captan, chlorothalonil, copper, folpet, mancozeb and sulfur) are contact fungicides.

Contact fungicides:

- must be applied *before* the spores land on and infect leaves;
- prevent spore germination, so they are preventive treatments; and
- have no effect once the infection is established.

Penetrant Fungicides

Fungicide mobility is influenced by many factors. Formulation impacts how a plant adsorbs or absorbs a product (see 'Surfactants' section in Chapter 7, this volume), but how a product performs is influenced by plant age, plant structure (e.g. architecture, cuticle hydrophobicity, trichome structure, etc.) and the environment, not just the physiochemical properties of the AI. *Penetrant* fungicides are absorbed, so they move or redistribute into plant tissues and penetrate beyond the cuticle and into the mesophyll of the treated leaf tissue itself. This redistribution exists on a continuum, and this continuum can be manipulated to a certain degree with formulation. As a result, there are various kinds of penetrants, characterized by their ability to redistribute when absorbed by the plant, and these products exhibit aspects of localized or translaminar movement. *Localized penetrants* remain in the area of initial plant contact and undergo very little movement within the plant (a process called translocation).

This absorption/penetration/uptake continuum includes:

- translaminar movement;
- xylem redistribution; and
- phloem/amphimobile movement.

For a fungicide to be considered *systemic*, it must be absorbed and translocated by the plant. All penetrant fungicides are systemic, because they are absorbed by the plant and translocated to other plant tissues. To be fully systemic, a fungicide must be translocated beyond the point of contact, into the vascular system, and distributed into the new growth of leaves, stems and roots. For fungicides, *systemicity* describes the uptake, transport and distribution of the AI within the plant via the xylem (Table 8.1). Movement can vary from nanometres to millimetres and even centimetres from the site of contact. The degree of fungicide translocation *in planta* is impacted by external and internal plant barriers such as the cuticle, trichomes and lignification of tissue and by the composition of the AI.

Some xylem-mobile compounds may redistribute into the phloem, cross cell membranes and move *symplastically* via a concentration-dependent gradient, assisted by cytoplasmic streaming. This also results in transfer *basipetally* (inward from the shoot and root apexes to the main stem) from the site of application. Systemic fungicides can be further subdivided based on the direction and degree of movement once they have been absorbed and translocated inside the plant:

- *Xylem-mobile fungicides* (also called *acropetal penetrants*) move upward from the point of entry through the plant's xylem. *Locally systemic fungicides* (essentially synonymous with localized penetrants) have limited translocation from the application site.
- *Amphimobile fungicides* (also called true systemic penetrants) move throughout the plant through its xylem and phloem. To date, only the phosphorous acid derivatives are true systemic fungicides.

Systemic fungicides can stop or slow infections within 72 h of exposure; this time period is modulated by host susceptibility and the degree of fungicide resistance by the isolate infecting. To be most effective, these fungicides must be applied soon after initial infection to arrest fungal growth. It is important to stress that all fungicides have limited curative activity.

Translaminar fungicides are absorbed by leaves and can move through the leaf to the opposite surface they contact but are not truly systemic and do not move throughout the plant. Strobilurin (QoI) fungicides all possess varying degrees of translaminar ability (Bartlett *et al.*, 2002) (Fig. 8.1). This vapour-phase systemicity

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Table 8.1. Mobility and classification of commonly used fungicides. (Authors' own table.)

Group code[a]	Fungicide family[b] or class	Common name	Example trade name(s)	Mobility
1	Benzimidazole or methyl benzimidazole carbamate (MBC)	Thiophanate-methyl	Topsin M®, Cleary's 3336®	Xylem-mobile
2	Dicarboximide	Iprodione	Rovral 4F, Chipco 26GT®, Iprodione	Localized penetrant/translaminar
3	Demethylation inhibitor (DMI)	Bayleton	Rubigan®, Strike®	Xylem-mobile
		Metconazole	Tourney®	
		Myclobutanil	Eagle®, Rally®	
		Propiconazole	Banner Maxx®, Tilt®	
		Tebuconazole	Indar, Tebuzol 250®, Torque®	
		Triflumizole	Procure®, Terraguard®	
		Triforine	Funginex®, Saprol®	
		Triticonazole	Trinity®	
4	Phenylamide (PA)	Mefenoxam	Ridomil, Subdue Maxx®	Xylem-mobile
5	Amines, morpholines	Piperalin	Pipron®	Non-mobile
7	Succinate dehydrogenase inhibitors (SDHI) – carboximides	Benzovindiflupyr	Aprovia, also mix partner of Mural®	Locally systemic
		Boscalid	Mix partner of Pageant®, Pristine®	
		Fluopyram	Luna Privilege, mix partner of Luna Sensation®, Broadform®	
		Flutalonil	Prostar®	
		Fluxopyroxad	Sercadis, mix partner of Merivon®, Orkestra®	
		Penthiopyrad	Fontelis®	
9	Anilopyrimadine (AP)	Fludioxanil + cyprodinil	Palladium®	Slightly mobile
11	Quinone outside inhibitor (QoI) – strobilurins	Azoxystrobin	Quadris®, Heritage®, Amistar®	Xylem-mobile
		Fenamidone	Fenstop®	Locally systemic/translaminar
		Fluoxastrobin	Disarm®	Locally systemic/translaminar
		Kresoxim-methyl	Sovran®, Cygnus®	Locally systemic/ translaminar
		Trifloxystrobin	Flint®, Compass®	Locally systemic/translaminar
		Pyraclostrobin	Cabrio, Headline	Locally systemic/translaminar
12	Phenylpyrrole (PP)	Fludioxonil	Medallion®, also a mix partner of Palladium®	Contact
14	Aromatic hydrocarbon (AH)	Dicloran	Botran 70®	Contact
		Etridiazole	Terrazole®, Truban®	
17	Hydroxyanilide	Fenhexamid	Elevate®, Decree®	Locally systemic

Continued

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Table 8.1. Continued.

Group code[a]	Fungicide family[b] or class	Common name	Example trade name(s)	Mobility
18	Antibiotic streptomyces	Streptomycin	Agri-Mycin®, Agri-Step®	Xylem-mobile
19	Polyoxin	Polyoxin D	Endorse®, PhD®	Xylem-mobile
21	Cyanoimidazole	Cyazofamid	Segway®	Low to slight
21(P)[c]	Host plant defence inducers, systemic acquired resistance (SAR)	Acibenzolar-*S*-methyl	Actigard®	Amphimobile
		Harpin	Messenger®	
28	Carbamate	Propamocarb	Banol®	Xylem-mobile
40	Cinnamic acid	Dimethomorph	Forum, Stature DM®, Stature SC®	Localized penetrant/translaminar
	Mandelic acid	Mandipropamid	Revus, Micora®	
45	Quinone inhibitor	Ametoctradin	Orvego®	Low to slight
M	Multi-site activity – chloroalkythios	Captan	Captan®	Contact
	Multi-site activity – chloronitrile	Chlorothalonil, chlorothalonil + propiconazole	Bravo®, Daconil 2787®, Concert II®	
	Multi-site activity – dithiocarbamate	Mancozeb	Dithane®, Roper®, Penncozeb®, Neotec®	
	Multi-site activity – inorganics	Copper	Champ FL®, Kocide®	
		Sulfur	Microthiol Disperss®, sulfur	
M (33)	Multi-site activity phosphonate	Fosetyl-aluminum	Aliette®	Amphimobile
		Phosphorous acid	Alude®, BioPhos®	
U (15)	Piperidinyl	Oxythiapiprolin	Orondis®, Segovis®	Non-mobile
U (28)	Unknown carbamate	Propamocarb	Banol®	Xylem-mobile

FRAC, Fungicide Resistance Action Committee; EPA, US Environmental Protection Agency; MOA, mode of action.
[a]FRAC code is listed in parentheses under the EPA group code when the codes differ. Neither system includes biofungicides.
[b]For the sake of consistency, group codes, fungicide classes, fungicide names and abbreviations are those used by FRAC and by EPA Office of Pesticide Programs. This programme is part of the pesticide classification system developed to assist growers in resistance management.
[c]Although similarly described, the MOAs are different.

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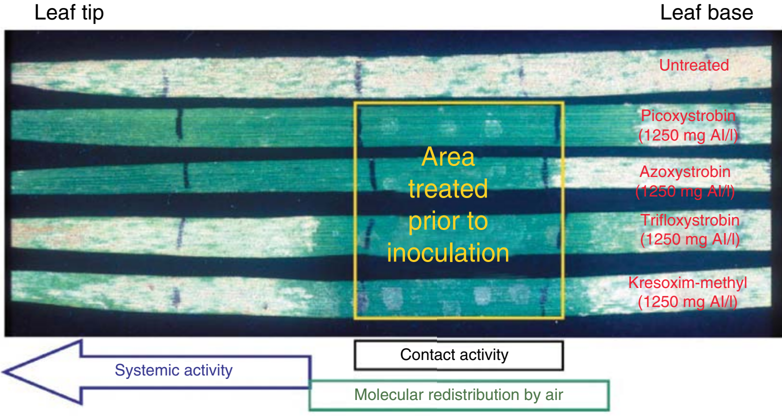

Fig. 8.1. Redistribution of strobilurins in wheat to control powdery mildew. (From Bartlett *et al.*, 2002.)

of this class of fungicides results from molecular redistribution by gas and is dependent on volatility and hydrophobicity (lipophilicity) of the AI (Fig. 8.2). Some strobilurins, like azoxystrobin and picoxystrobin, are both translaminar and xylem-mobile. Lastly, redistribution is a function of mass transport, particularly diffusion and osmosis. One consequence of this is that some products may seem to act systemically at different (higher versus lower) application rates.

Chemical Characteristics of Commonly Used Fungicides

For drug discovery, *Lipinski's Rule of Five* identified the physiochemical and structural properties of bioavailability for drug candidates and reduced the search parameters to four basic molecular descriptors based upon a set of known drugs (Lipinski *et al.*, 2001). For a compound to be considered 'drug-like', descriptors include:

1. A molecular weight less than 500 Da.
2. The number of hydrogen bond donors ≤5.
3. The number of hydrogen bond acceptors ≤10.
4. An octanol–water partition coefficient (K_{ow}, also referred to as *P*) that does not exceed 5.

Note that Lipinski's Rule of Five is composed of four rules that are composed of multiples of 5; like most heuristics, there are many exceptions. In fact, more than 15% of the drugs surveyed did not meet all the listed criteria (Lipinski *et al.*, 2001). Tice (2011) was the first to modify this approach for agricultural chemicals, but did not include fungicides in his study, which is curious as fungicide bioactivity compares favourably with Lipinski's Rule of Five: most fungicides have a molecular weight of less than 500 Da (Table 8.2) and most fungicides possess an octanol–water partition coefficient (K_{ow} or *P*) less than 5. The *octanol–water partition coefficient* K_{ow} describes the relative distribution of the AI between 1-octanol (which mimics a hydrophobic bilayer) and water and is a measurement for *lipophilicity* (Hermens *et al.*, 2013; Harris and Logan, 2014). The K_{ow} values of organic compounds span orders of magnitude; log transformation of K_{ow} (log K_{ow}, also called log *P*, denoting lipophilicity) values are typically between −3 (very hydrophilic) and +10 (extremely hydrophobic).

Unlike drugs that are injected or ingested, fungicide mobility is determined by the interplay between *lipophilicity* to permeate biological barriers (e.g. epicuticular waxes, cuticle, internal plant membranes and the hydrophobic fungal cell walls and membranes) and *solubility*, which is necessary for within-plant transport (Klittich, 2014). Solubility is a function of a solute to dissolve to form a homogeneous solution. In this way, the partition coefficient (K_{ow}/log *P*) measures the solubility of a compound in a hydrophobic solvent (octanol) and a hydrophilic solvent (water). The logarithm of these two values enables compounds to be ranked in terms of hydrophilicity (or hydrophobicity). Solubility is

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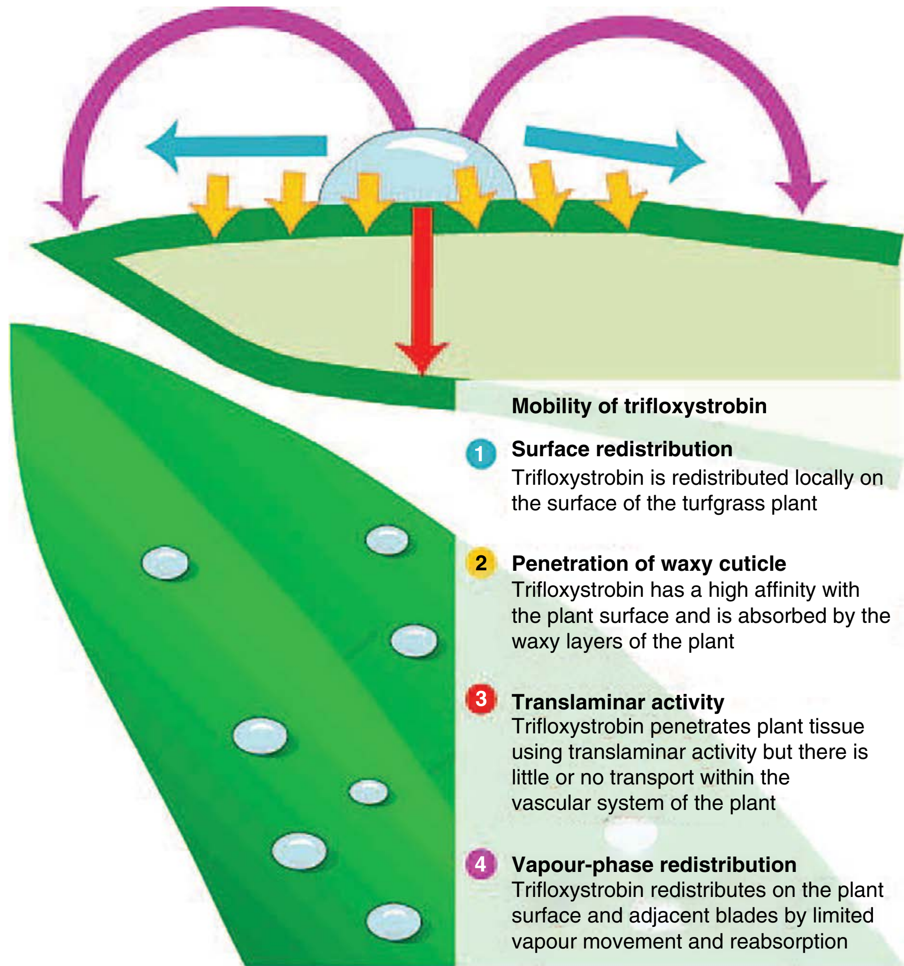

Fig. 8.2. Mobility of trifloxystrobin. The dual issues of coverage and redistribution of the fungicide intersect. Strobilurin fungicides have a zone of efficacy that extends beyond the droplet–cuticle interface. This redistribution of a fungicide occurs over the leaf surface via re-wetting. (Image used with permission by Bayer Crop Science.)

also a function of the *acid–base dissociation constant*, pK_a. The pK_a indicates the strength of an acid and identifies the pH that results in an equal concentration of ionized and non-ionized forms of the pesticide. The pH of the solution and the pK_a of the pesticide determine the proportion of ionized to non-ionized pesticide molecules, as per the Henderson–Hasselbach equation. Because fungicides can be contact or systemic, hydrophobicity is not necessarily a key determinant in efficacy. In fact, contact fungicides are often lipophilic, readily adsorb to the waxy plant cuticle and persist due to their lipophilic nature. Unlike pharmacology, fungicides can be formulated to circumvent solubility issues, through the use of suspended colloids (SC) and emulsifiable concentrates (EC) formulation.

Understanding fungicide mobility is essential for obtaining the best results from any fungicide application and mitigates those factors (environment, timing, coverage) that can render an application ineffective.

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Table 8.2. Basic chemical characteristics of commonly used fungicides. (All data derived from PubChem.)

Fungicide	Chemical class	Water solubility (mg/l)	Log K_{ow} [a]	K_{oc} [b] (l/kg)	Soil half-life (days)
Azoxystrobin	Strobilurins	6 at 20°C	2.5 at 20°C	500	56
Captan	Phthalimide	5.1 at 25°C	2.8	33–600	5.5–20
Chlorothalonil	Dinitrile	0.81 at 25°C	3.05 at 20°C	900–14,000	10–40
Cyprodinil	Anilinopyrimidines	13 at 25°C	4.0 at 25°C	1,706	20–60
Fludioxonil	Phenylpyrroles	1.8 at 25°C	4.1 at 25°C	75,000	140–350
Fluopyram	SDHI	16	Unknown	319–591	57–1757
Fosetyl-al	Phosphorous acid	111,300 at 20°C	–2.1 to –2.7 at 23°C	20–311	<1 to <1.8 h
Mancozeb	Dithiocarbamates	6.2 at 25°C	1.3	1,000	1–7
Metalaxyl	Phenylamides	8.4 at 22°C	1.8 at 25 C	30–300	10–40
Myclobutanil	Triazoles	142 at 25°C	2.94	950	66
Penconazole	Triazoles	73 at 25°C	3.7 at 25 C	2,205	133–343
Polyoxin D	Polyoxins	8.6	Unknown	999	999
Procymidone	Dicarboximides	4.5 at 25°C	3.1 at 26 C	378	28–84
Propiconazole	Triazoles	0.1 at 20°C	3.7 at 25 C	950	29–70
Pyraclostrobin	Strobilurins	1.9 at 20°C	3.99 at 22°C	6,000–16,000	2–36
Pyrimethanil	Anilinopyrimidines	121 at 25°C	2.8 at 25 C	265–751	7–14
Quinoxyfen	Quinolines	0.116 at 20°C	4.7 at 20°C	15,415–75,900	11–454
Tebuconazole	Triazoles	36 at 20°C	3.7 at 20°C	769	40–170
Vinclozolin	Dicarboximides	2.6 at 20°C	3.1	100–735	34–94

[a] Bromilow and Chamberlain (1989) define log K_{ow} values as follows: –3 to 0 as hydrophilic; 0 to 3 as intermediate; and 3 to 6 as lipophilic.
[b] K_{oc} is the organic carbon–water partition coefficient and is a measure of a chemical's mobility in the soil. High values mean the substance is highly adsorbed to soil and is unlikely to leach into groundwater; low values mean the substance is mobile in the soil. K_{oc} is often estimated from the K_{ow}.

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9

Application and Sprayer Technology

Key Points

- Application methods include seed treatment, granular incorporation, foliar application, 'sprenches', drenches, dips and injection.
- Optimal pesticide application is defined as the deposition of the most biologically efficacious dose on the target combined with minimal health or environmental impacts and maximum economic sustainability.
- Fungicidal control of crops grown indoors (glasshouses, hoop houses, etc.) needs special care during application.

Introduction

For fungicides, we define optimal pesticide application as the deposition of the appropriate dose on the target combined with minimal health or environmental impacts and maximum economic sustainability (Matthews, 2014). Fungicides may be applied to crops as pre-planting granular applications, seed treatments, drenches, foliar sprays, postharvest dips, or even injected into the plant. The primary objective of all fungicide applications is the delivery of a uniform dose of fungicide in a safe and timely manner. Product that fails to be captured by the target results in a loss of efficacy. It can also result in drift, leakage, runoff and spill, all of which have significant ramifications to the health of the applicator, other people and the environment. No technology can compensate for a poorly trained or distracted operator. The applicator is the sole common denominator for all these treatment methods; this person must be competent, careful and cautious for an application to be successful.

Application Types

Seed treatments

The first application of fungicides involved the use of seed treatments by Tillet, in 1755 (see 'The History of Fungicide Use' section in Chapter 1, this volume). Fungicide seed treatments are of increasing importance in the establishment of many crops and will only increase as farmers expend more in expensive hybrid and transgenic seed, to protect their investment. It is important to stress that fungicide seed treatment cannot compensate for poor seed quality and that good cultural practices (e.g. deploying resistant or tolerant cultivars, using pathogen-free seed, proper fertilization practices and improving soil drainage) are all part of an IPM strategy. The term 'seed treatments' is taken to include corms, rhizomes, bulbs, tubers as well as true seeds and involves synthetic chemicals, BCAs, biologicals and basic substances. Seed treatments provide

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pesticide availability when needed, with little or no risk of drift or non-target site applications.

Fungicide seed treatments target seed-borne pathogens, including the smuts and bunts of cereal crops, along with soil-borne pathogens including those that cause pre- and post-emergent damping-off (McMullen and Lamey, 2000; Babadoost and Islam, 2003). The most common pathogens targeted for seed treatment include the oomycetes *Pythium* and *Phytophthora* spp., along with the fungal pathogens *Rhizoctonia* spp., *Phomopsis* spp. and *Fusarium* spp.

Seed treatment improves crop establishment in reduced-till or no-till fields and provides insurance when earlier planting dates coincide with cool and wet soils that expose seedlings to damping-off. Seed treatment is essential for those crops where seeds are known to have poor germination rates or are slow to emerge (Stone *et al.*, 1987; Lamichhane *et al.*, 2020). In potato, seed tuber treatment is correlated with wound compartmentalization and prevents cut pieces from sticking together. Phytotoxicity of treated seed may also be an issue especially if there are long delays between applying the fungicide and planting the seed (Khaleeq and Klatt, 1986).

Historically, the use of seed treatments was confined to immobile fungicides such as the organomercurials. Nowadays, seed treatments are seen as a convenient and economic way to apply systemic fungicides. Seeds can be coated, dressed, encrusted or pelleted, but thorough coverage is essential for complete protection (Fig. 9.1). One drawback to seed treatment is that dose is limited to what adheres to the treated seed. There is considerable interest in the use of slow-release technologies for the application of systemic fungicides as a seed treatment to provide long-term control of crop disease because fungicide seed treatments do not normally maintain their efficacy beyond the seedling state. Formulation technology (slow or timed release) of seed coatings is improving, but phytotoxicity remains a problem due to the fragile nature of seedlings.

Fig. 9.1. Treated seed can be dusted, encrusted or pelleted. (© BASF used with permission.)

Seed treatment formulations are just as complex as their foliar counterparts, and include dry flowable (DF), dusts (D), flowable (F), flowable seed treatment (FS), liquid (L), liquid suspension (LS) or wettable powder (WP) formulations (see Chapter 7, this volume). Seed treatments commonly contain adjuvants, surfactants (dispersants) and inert substances to adhere fungicides to the seed surface and to reduce or eliminate dust. They must adhere to the seed, but not ball up or cause bridging in the hopper box or damage the planting equipment. Some slow-release formulations deliver the AI over several weeks using binders, clays, fillers or polymers. Seed treatments often contain a coloured dye to distinguish them from untreated seeds. An overriding consideration is that the seed treatment does not interfere with the process of germination.

To achieve these different criteria, seeds can be coated in different ways. Dusting is the simple application of fungicide dust or powder (usually a few grams of fungicide per kilogram of seed). Film coating uses a thin layer of binder, often a clay but more recently these have included gum arabic, ethyl- and methylcellulose, polyethylene glycol and chitosan (especially for biologicals). For smaller or more valuable seeds, encrusting the seed in a coat of fungicide, along with binders and adjuvants, preserves seed shape. Pelleting is similar to encrusting, but the coating applied results in loss of shape or structural

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detail and incorporates fillers such as clays, chalk and diatomaceous earth to provide bulk.

Seeds purchased from seed suppliers are treated in bulk prior to packaging and delivery. Many companies and seed distributors treat farmer-saved seed for individual growers, but in recent years, many farmers have purchased the machinery needed to treat seed. The decision to purchase seed treatment equipment is like any pesticide application: is it cost-effective to purchase a seed treater? What is treated? When does it need to be treated? How much capacity is needed? Economics would depend upon how many acres or seeds a grower is treating, what they are treating, what pathogen they are trying to protect against, and having the manpower and expertise to perform the treatment.

A diversity of machinery is available to treat seeds of all shapes and sizes. These include dust treaters (that are no longer commonly used) and metered slurry treaters where fungicide(s) is (are) applied as slurry that is measured via a slurry cup and seed dump pan. The cup introduces a given amount of slurry to each aliquot of seed into a mixing chamber where they are blended. Slurry treaters are adaptable to a diversity of seeds and different rates of treatment. The small amount of moisture added to the seeds (less than 1% seed weight) does not affect seed in storage, since the moisture is applied to the seed surface and is quickly lost.

More modern seed treaters include computer-controlled proportional meters to ensure the appropriate treatment rate is applied. The seed is first augered using a helical drill into a tilting dump pan. Upon reaching a pre-set weight, the pan tilts to one side and dumps the seed. The dumping of the pan initiates the dumping of a cup of seed treatment via mechanical linkage(s), making treatment application proportional to the flow rate of the grain. With these systems variations in the seed flow rate do not affect the application rate, providing consistent application. Regardless of the type of equipment chosen, careful calibration is necessary to apply the appropriate treatment on the correct weight or volume of seed for the treatment to be effective. Despite the overall reduction in pesticide use because of seed treatment, potential environmental problems remain. Studies with insecticides found that approximately 5% of the substance reaches its destination, the cells of the plant, with 1% getting blown off as dust; the remaining 94% ends up in the soil or groundwater (Gross, 2014). Finally, treated seed runs the risk of being ingested by birds or other animals; care must be taken to protect both seeds and seed predators, particularly if seed treatments include insecticides.

Granular application

Granular application provides an efficient mechanism for applying pesticides to the crown or roots of susceptible plants, assuming the roots grow towards the fungicide and adsorb/absorb it. In most instances, the dosage of AI in granular pesticides is lower than in foliar applications. This is because less fungicide is needed to protect the seed or seedling than a mature plant. The fragile nature of seedlings also necessitates a reduced dose to minimize any phytotoxicity.

Just as there are many variables involved in delivering a liquid formulation to a target, there are many variables involved in granular fungicide applications. In the greenhouse or nursery, granular applications are incorporated into the planting medium with a cement mixer or other mixing apparatus to protect against soil-borne pathogens. Granular pesticides can be applied by rotary or drop spreader in turf, landscape or nurseries. Some ready-to-use (RTU) granular products are designed so that they can be shaken out of the package without requiring any special application equipment. With row crops, a banded application can be performed below the seed or on the soil surface after planting. A banded fungicide application involves treating planted rows, and not applying pesticide in between rows or alleys. This results in less pesticide applied, distinguishing it from broadcast application over an entire area. Just like any application, calibration must be performed on the spreader with the product that will be applied. Lastly, in-furrow application of granular fungicides is very common at planting to protect many seeds and seedlings of vegetables, bulb crops, cotton and groundnuts from an assortment of seedling diseases. Granular fungicides are essential tools for fungicide application in smallholding rice production, for control of both rice blast and sheath blight during flooded phases. With all

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granular applications, the applicator needs to ensure that the product is thoroughly mixed with the surrounding soil. Rainfall or irrigation (or even a heavy dew) is necessary for granule dispersion and fungicide release into the surrounding medium or soil.

Pre-planting dips

The use of pre-planting dips to eradicate pests is commonly employed in the vegetable industry for bulbs, tubers, corms and crowns, and in the ornamental industry where this practice is expanded to cuttings and whips. Dipping these propagative units prior to planting significantly reduces the risk of pathogens being introduced to a field or growing facility.

For most pesticides, it is important to remember that dip rates are almost always lower than spray rates to prevent phytotoxicity, particularly when dipping cuttings. Many (but not all) of the fungicides used in dips are protectant, so total coverage of the bulb/corm/tuber or cutting is required. It is important to ensure full coverage of the plant propagule by the fungicide dipping. Only intact healthy bulbs, corms, tubers or cuttings should be dipped as there is a risk of spreading infection. Good sanitation is important to ensure that dipping does not result in bacterial disease issues, particularly from bacterial soft rots of the genus *Pectobacterium* (*Erwinia*). The temperature of the dip solution (Daines, 1970), stage of the plant and time to drying all impact the efficacy of the dip treatment.

Dips have been shown to enhance both plant vigour and fruit production and can reduce the number of foliar applications needed later in the season (Dong *et al*., 2013). None the less, foliar applications may still be required for continued protection against subsequent infections (e.g. Daugovish *et al*., 2009) (Fig. 9.2).

Postharvest dips

There is still a need to protect a crop after it has been harvested and delivered to a storage facility and fungicide dips play a large part in this. Losses on various crops can be 50% in the absence of adequate storage facilities and fungicides (Coursey and Booth, 1972; Kanetis *et al*., 2007), and even higher in developing countries without access to either (Smoot *et al*., 1971). Fresh produce, which includes leaves, fruit, stems, roots and tubers, is highly perishable to decay and deterioration.

Many postharvest rots are opportunistic wound pathogens, including the green and blue moulds, caused by *Penicillium digitatum*, *Penicillium expansum* and *Penicillium italicum*, along with *Botrytis* spp., *Colletotrichum* spp., *Botryosphaeria* spp. and the zygomycetes *Mucor* spp. and *Rhizopus* spp., to name but a few. All these fungi share the ability to infect fruit in the orchard, grove, field or packinghouse, and during distribution. They reproduce rapidly, their spores are ubiquitous and can survive long periods of time on a variety of surfaces including bins, crates and storage facilities.

An integrated approach for postharvest fruit management includes minimizing injury to produce during the harvesting and storage process, and sanitation, such as cleaning immediately postharvest using soap or other detergent with or without sodium carbonate (Na_2CO_3) or sodium bicarbonate ($NaHCO_3$) to remove residues. Organic acids, including sodium benzoate and potassium sorbate, are also used, and combined with fungicides. The use of fungicides is commodity-dependent and may be prohibited in some countries or limited to just a few registered chemicals. To date, postharvest fungicides include azoxystrobin, fludioxonil, imazalil, pyrimethanil, sodium ortho-phenyl phenate, thiabendazole, trifloxystrobin, or mixtures of these and other compounds. Not surprisingly, given the nature of these pathogens, fungicide resistance is an issue (Kanetis *et al*., 2007).

Biological control of postharvest diseases via bacteria, yeasts and other fungi has been a focus of recent research. BCAs can protect against wound pathogens infecting injuries that occur during harvest and transport. This assumes the BCA can colonize, survive and reproduce in the storage environment while not otherwise impacting product safety, quality or flavour. To date, despite many commercialization attempts, the lack of a successful commercial product belies the early optimism surrounding this approach for postharvest disease control, and the search for better biologicals continues.

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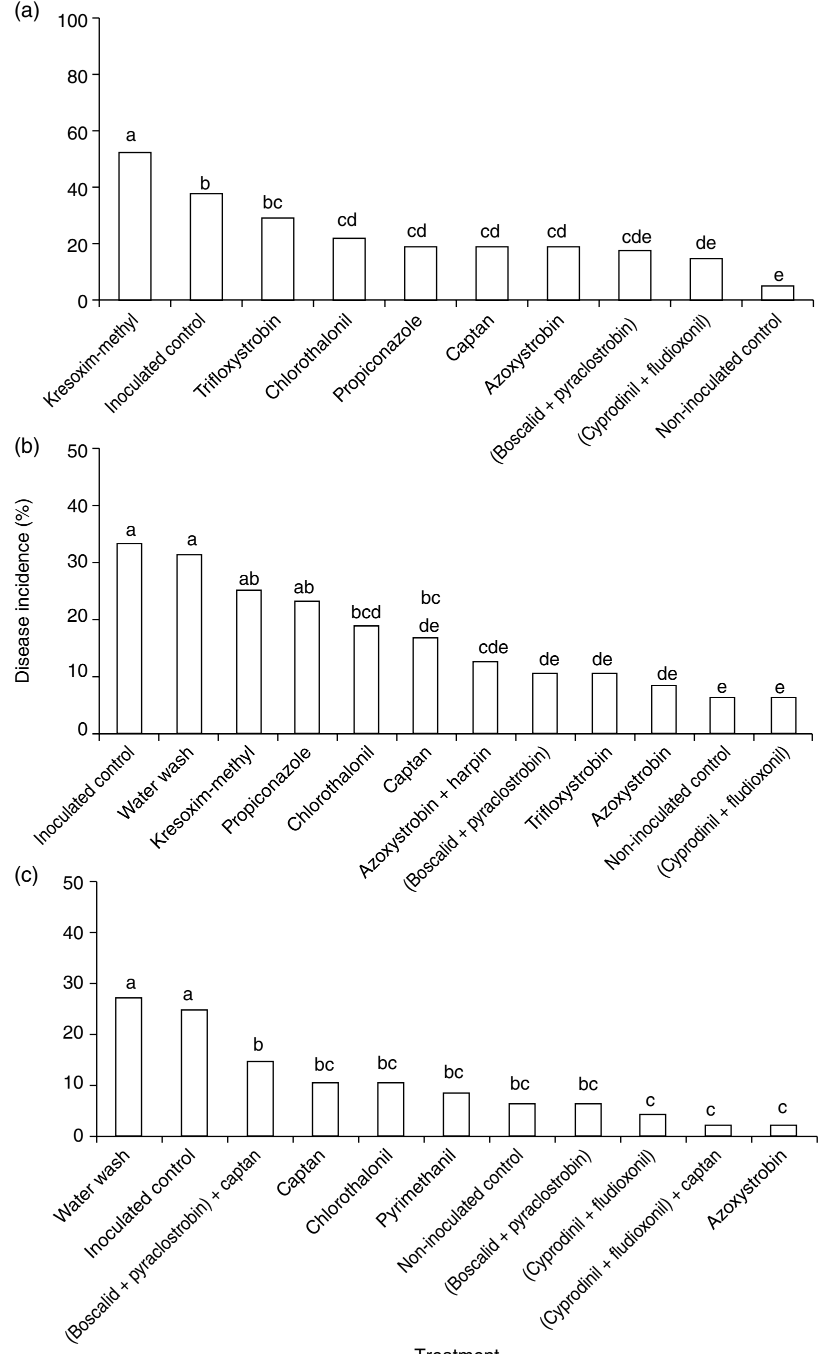

Fig. 9.2. Disease incidence of strawberry plants at 2–3 weeks after planting for 'Baeza' in summer 2002 (a), and 'Camarosa' in winter 2002 (b) and 2004 (c), inoculated with *Colletotrichum acutatum* and dipped in fungicides or washed with water at Oxnard, California. Treatments with unlike letters were significantly different from each other according to Fisher's protected least-significant difference test at P = 0.05. (From Daugovish *et al*., 2009.)

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Fungicide injection

The long-lived and perennial nature of trees, coupled with the inefficiency of ground or air applications for their protection, prompted investigations into the injection of all types of pesticides into trees. Tree injection was first document in AD 1158; even Leonardo da Vinci apparently conducted experimentation on the use of arsenic in peach trees to make fruit poisonous (Roach, 1939). Much of this early work, as observed by Roach (1939), 'though interesting historically, appear[s] to have been the results of more or less inspired guesses, and the efficacy of most of the methods used may well be doubted; but the idea behind them, like that behind modern injection work, was to improve the health of the plant or to bestow on it special qualities without either killing or even unduly damaging it.' Recent work has done little to improve upon this.

Currently, injection is used only for high-value trees due to several issues, chief among them being cost. For arborists, fruit and nut growers, fungicide injection serves as a grail of sorts, in that it possesses the potential to improve efficacy and provide direct delivery to the target while preventing unnecessary environmental impact. Not surprisingly, injection as a delivery method has increased with new developments in pesticide formulation technology, injection methodology, new devices and increased availability of off-label compounds. Injection is regularly promoted as a means of reducing the risk of exposure of the public, applicators, the environment and wildlife to both pesticides and pesticide drift. However, therapeutic treatment as an option is limited to the early stages of invasion (less than 20% symptom development in the canopy) in a few landscape tree species (Perry *et al.*, 1991; Haugen and Stennes, 1999) and has been looked at in orchards and vineyards. It is not a silver bullet, but it can serve as a potentially effective tool when added to a full IPM programme. The cost of treatment may be offset by the cost of tree removal, or the value that mature overstory trees give to landscapes in terms of aesthetics and ecosystem services.

These interests drive work into the possibility of tree injection of fungicides. Unfortunately, tree injection research, unlike other aspects of fungicide research, has often displayed very little rigour. As a result, marketing and hype drive the field and often promote systems and practices that are marginally effective at best, or even damaging to long-term tree and ecosystem health, at worst. Tree injection for disease control has been demonstrated to be a valid principle and practice for the mitigation of Dutch elm disease, oak wilt, laurel wilt and Ramorum canker for high-value landscape trees. Unfortunately, for wider-scale deployment of fungicides for plant protection, there is a lack of scientific reproducibility, guidelines and, most importantly, economic analysis.

Drenches

Both drenches and soil injections involve the delivery of dilute, liquid fungicides into the soil (or soilless medium in the case of nurseries and greenhouses) proximal to the roots. Chemicals must be water-soluble for root uptake. Ideally, applications should be made to moisten but not saturate the medium or surrounding soil. Fungicides should not be applied to dry soil. Application rates usually depend on the size of the plant (from pot size in the greenhouse to diameter breast height (DBH) for trees) and are usually much less than for foliar applications, as the roots are more sensitive to exogenous chemicals. Soil injections are a variation on drenches and are far less common for fungicides than insecticides. Soil injectors pressure-inject fungicide below the soil, usually near the main stem of mature trees. On the opposite side of the spectrum, subsurface drip chemigation relies upon gravity to deliver fungicides to roots.

Drenches, sprenches (Box 9.1) and even injections are effective methods of protecting plants from infection by root rot fungi (Stone *et al.*, 1987; Meyer and Hausbeck, 2013). They do not cure infected plants but protect plants from infection in the first place. Multiple studies have demonstrated that soil drenches for root and crown rots are more effective than foliar applications. Similarly, studies show chemigation is an effective method of fungicide delivery for root rot disease management. However, drenches, particularly those containing thiophanate-methyl, flutolanil or QoIs, inhibit a broad range of fungi and may interfere with mycorrhizal associations. Finally, soil drench uptake interacts with soil type, structure and soil water capacity, with

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Box 9.1. Sprenches.

Typically, when a spray application is applied, the target is the foliage, with a greater proportion of the application reaching the crop rather than the roots and soil below. However, for some pathogens that exist in roots, crowns and leaves, an application that addresses all three would be expected to provide the best control. Sprenches (a portmanteau of spray and drench) are an unsanctioned reality of specialty crop growers that address all phases of certain pathogens that exist as foliar blights, crown and root rots, like some members of the genera *Pythium*, *Phytophthora* and *Rhizoctonia*. Sprenches often provide greater control with fewer applications and may explain why smaller plants are often better protected by fungicides than larger plants, due to an unintentional sprench effect during application (although many other factors are probably at play here). When comparing a spray to runoff (0.5% w/v rate), mist application (10% w/v rate) and foliar application (1% w/v rate), the spray to runoff (sprench) was comparable to the mist treatment (20-fold higher rate); the 1% w/v foliar application compared as well as the 20 and 40% w/v mist application (Fairbanks *et al*., 2000).

Sprenches are commonly used in specialty crop production, where the higher value of the commodity and labour inputs may justify the price of the application. Vegetable transplants, seedlings, plugs and cuttings are both more delicate and smaller than mature plants, necessitating different application techniques to ensure coverage while preventing mechanical damage via the spray application or equipment. Reducing sprayer pressure reduces the risk of damaging transplants, plugs and seedlings or of dislodging unrooted cuttings. However, the lower volume often results in larger droplets that run off the plant and also drench roots. Sprenches often have a longer period of activity than sprays, resulting in fewer applications. This can reduce the labour hours needed to keep a crop protected. Currently, labels are not written to take account of the action of drench-spraying that often occurs during application.

some clay soils potentially binding fungicides and reducing efficacy. Light sandy soils may also prevent absorption of the fungicide as the solution moves through the soil profile.

Soil injection or drench methods involve placing chemicals in liquid form near the roots in the soil for root uptake. As with the other injection methods, the chemicals must be water-soluble. Chemicals should be applied to moist, but not saturated, soil. With the soil drench method, the chemical is mixed in water and poured on to the soil near the tree's root crown. Mulch or other surface organic matter is pulled back, the chemical is poured directly on the soil and then the mulch is replaced. The amount of chemical used is based on trunk diameter and will be stated on the label. Soil injection methods vary somewhat, but typical recommendations are to inject chemicals 5–10 cm deep with a high-pressure injector either within 45 cm of the trunk or on a grid.

Foliar treatments

The most important target of fungicide application is to the foliage of crops. Given that the primary objective of spraying a fungicide is to increase profitability, it is necessary to consider the following:

- timing – when should the first application be made to prevent infection?
- canopy structure – early-season applications often require less volume to achieve adequate coverage, whereas later-season, denser canopies may intercept the product before it can reach the targets (i.e. the photosynthetically active leaves and the fruit to be harvested).
- rate of application – how much fungicide is required?
- number of applications – how often must the plant be sprayed?
- equipment – what type of sprayer must be used, and what type of nozzle is necessary to create effective delivery of application?

Most foliar fungicides are diluted in water before application (aerial applications often employ oil). The mixture is delivered through atomizing nozzles operating under high pressure that are designed to disperse fine droplets of the product evenly throughout the crop. The two most important factors for successful foliar fungicide application are that: (i) the droplets adhere to and do not run off the leaf surface; and (ii) the

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fungicide is distributed uniformly over the plants. To do this successfully, the volume of diluted fungicide will vary according to the crop, the developmental stage of the crop and the activity of the product. Successful applicators recognize that droplet size, nozzle type, operating pressure, formulation and target are interdependent variables in the application of fungicides (Nuyttens *et al.*, 2007, 2009).

When applying foliar pesticides, the field that was visualized as a flat, two-dimensional area now becomes a three-dimensional one. The dose or volume of spray being recommended is contingent upon the growth or developmental stage of the plants within the field – overlooking this third, vertical dimension can result in significant underdosing of crops. When spraying foliage, rates should be applied relative to the area of plant surface present at the time of application, rather than the ground.

Using Fungicides on Protected Crops

The application of fungicides in greenhouses, hoop houses, warehouses, etc. (combined here as 'greenhouses') faces different issues compared with in-field applications, even when applied to the same crop. Pesticide labels address applications in enclosed places in one of two ways:

1. Pesticide labels may explicitly state that the product is registered for use in greenhouses. Pesticide labels may have different instructions for greenhouse use and in-field use, and these restrictions take account of the difference in cuticle development: greenhouse-grown plants have thinner cuticles than field-grown plants and are more prone to phytotoxicity. Other issues include weathering and time to dry. In some instances, these products may also be used in high tunnels, shade houses or lathe houses according to label instructions.
2. Pesticide labels may explicitly prohibit greenhouse use. These products cannot be used in a greenhouse under any circumstances. Reasons may be due to issues of phytotoxicity on the crop, human health, or an unwillingness of the manufacturer to assume the costs necessary to test safety of their product in an enclosed system.

Many pesticide labels do not specify whether the product can be used in a greenhouse or not. When labels do not explicitly prohibit greenhouse use, most US state regulatory agencies interpret that to mean the product can be used in a greenhouse so long as the treated crop is on the label and the product is used according to label directions. In the EU and other countries, this is interpreted to mean that the pesticide may not be used.

Sprayer Technology

To minimize the impact of pesticides in the environment, it is important to understand how pesticides are delivered. Pesticides are commonly delivered via a sprayer. Sprayers differ in how they spray, their volume, flow rates, pressure delivery and nozzle types. All these features must be matched to the respective crop to improve outcomes. Sprayer technology is always improving with computerized systems helping to calibrate speed and spray volume considerations. This technology improves application targeting, coverage (better and more uniform) and waste reduction (through smart-sprayer technology), leading to more efficient and effective use of pesticides.

At its most basic, a sprayer is simply a machine that distributes a product to a desired target. Modifying the droplet size and flow rate via the sprayer is the simplest, most economical approach to improve fungicide efficacy and efficiency. The processes involved in pesticide delivery and deposition are considerable, along with the variables that need to be controlled (Fig. 9.3). During this process, a sprayer must aerosolize the solution into droplets of a defined size, then deposit those droplets on the target with sufficient, but not excessive, pressure to penetrate the plant canopy and uniformly cover the target (which may consist of leaves, stems, flowers, fruit and/or roots) while not drifting to unintended targets or sites, volatilizing, running off the plant or shattering upon impact.

To do this, many different types of sprayers have been created, from small, spot-treatment sprayers, to crop dusters that cover large areas. However, all sprayers have the same basic components:

- *Tank*, the container that holds the chemical solution, usually composed of a chemically resistant material.

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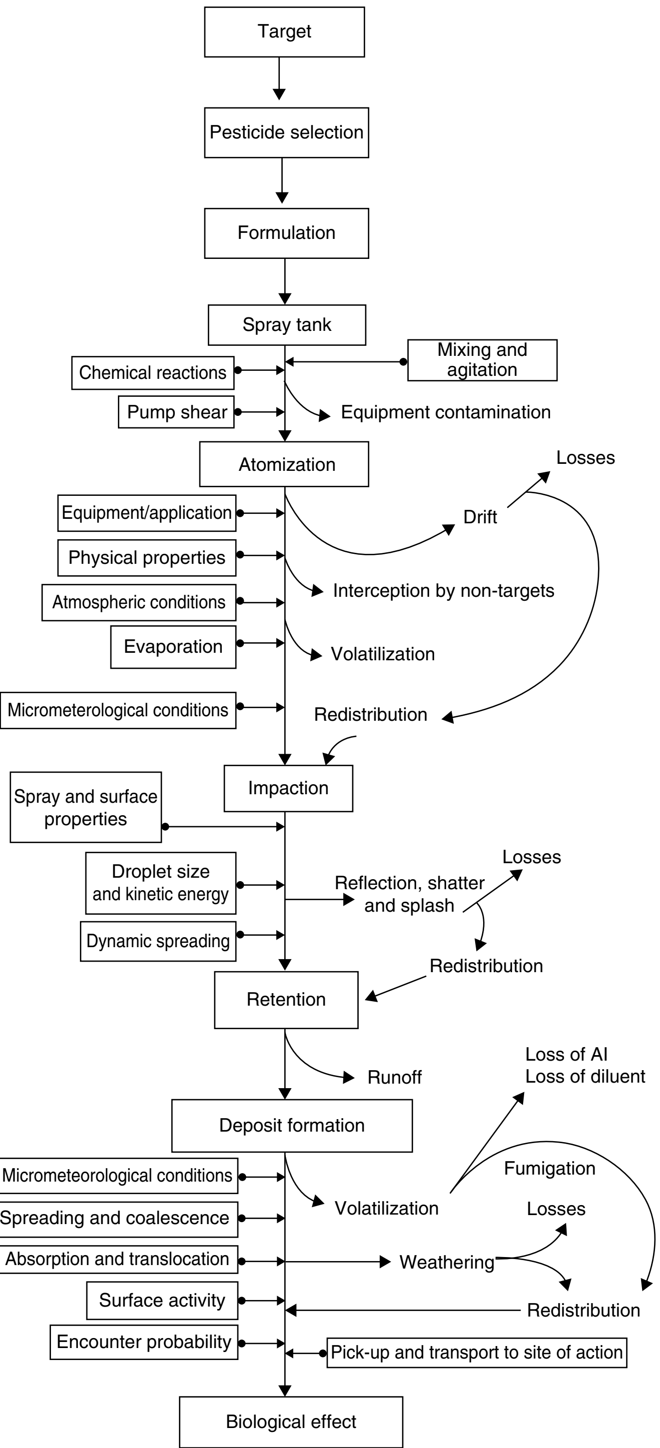

Fig. 9.3. Processes that factor into pesticide delivery and deposition. (From *Pesticide Application Methods*, Matthews, 2014, with permission.)

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- *Pump*, the component that moves fluids or gases by physical or mechanical action from one location to another.
- *Agitator*, to maintain the emulsions or colloidal suspensions in a homogenized solution. Agitators can be mechanical (paddle or propeller) or hydraulic and flow based.
- *Air chambers*, used in reciprocating pumps, that modulate pulsations and provide consistent nozzle pressure.
- *Pressure gauge*, to indicate the pressure used for pesticide delivery.
- *Pressure regulator*, to allow for pressure adjustment of the pump and may serve as a safety device in some sprayers by automatically releasing excess pressure.
- *Valves*, devices that regulate fluid flow by opening, closing or restricting various flowthroughs. There are three main types of valves:
  - *cut-off valve*, which controls pump flow to the delivery line;
  - *by-pass valve*, which permits the flow from pump to tank to bypass the delivery line; and
  - *relief valve*, which controls the pressure within a predetermined range.
- *Strainer*, which (as the name suggests) filters out dust or abrasives in the solution to reduce wear and abrasion to the sprayer. It is commonly a small, plastic ring with mesh (Box 9.2).
- *Suction hoses*, designed to withstand a vacuum and prevent hose collapse, while also being airtight, chemical- and abrasive-resistant. Ideally, they are as short as possible, and as large as the intake port.
- *Delivery line*, which channels the pesticide to the tip for delivery.

Box 9.2. Strainers and lines.

Many factors can go wrong with a pesticide application, but a plugged nozzle is by far and away the most aggravating (Fig. 9.4). Choosing the appropriate strainer and making sure it is correctly positioned reduces nozzle plugging and wear. Prior to ever reaching the nozzle, the pesticide is routed to the tank and strained via the basket, followed by the line strainer and the nozzle screen. Note that each level of straining gets progressively finer. Mesh is a measurement of particle size; in strainers, the higher the mesh number, the smaller the screen opening and the smaller the particle that will pass through. A basket strainer set in the tank-filling orifice strains the pesticide to prevent debris from gaining tank entry and prevents clots of wettable powder (WP) into the tank until they are dispersed. Basket mesh size is often 16- to 20-mesh. The next and most critical strainer is in the line. This screen/strainer should be cleaned regularly. Depending upon the formulation of pesticide used, the pesticide-line mesh size ranges from 16 to 80. The position of the line strainer varies upon the type of pump used in the sprayer; it can be positioned between the tank and the pump, between the pump and the pressure regulator, or close to the boom, depending upon the type of pump used. Roller and other positive displacement pumps should have a line strainer (40- or 50-mesh) located ahead of the pump to remove material that would damage the pump. In contrast, the inlet of a centrifugal pump must not be restricted. A line strainer (usually 50-mesh) should be located on the pressure side of the pump to protect the spray and agitation nozzles. A final strainer may appear in the nozzle, to maintain the spray pattern and keep the nozzle sediment-free, while preserving gaskets. These are particularly important when using dry formulations of pesticides.

Fig. 9.4. A nozzle assembly consists of the nozzle body, strainer and the nozzle tip (in this case the disc–core tip); this is secured by the cap. (From TeeJet Technologies, with permission.)

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A sprayer can be as simple as a bottle (tank) and the delivery line or tube that takes the liquid from the tank to the pump (Fig. 9.5). However, like all technologies, improvements and modifications are made to improve delivery. For a spray bottle, the pump is worked by hand via a trigger lever or mechanism, along with a piston, housed inside the cylinder. Within the cylinder are a spring and a valve; the last three compose a pump, specifically a positive displacement pump. The pump forces this liquid down the barrel and out a small hole at the spray valve. Gripping the trigger compresses the spring, driving the piston into the cylinder and forcing the liquid through the nozzle by displacement. Trigger release moves the piston and the liquid back into the cylinder. These two strokes of the piston, into the cylinder and out again, constitute the entire pump cycle. A one-way valve allows liquid to flow up the cylinder and into the pump, not back into the bottle. There may also be a strainer at this part of the tube to prevent abrasives from blocking a sprayer from working.

Multiple factors must be considered in choosing a sprayer. One of the first considerations is the size of the area to be sprayed. Topography can also impact sprayer choice, particularly for power take-off (PTO) driven sprayers that use a drive shaft attached to an engine to drive the sprayer or other machines. If using one sprayer for a diversity of crops as many vegetable and greenhouse growers do, the grower needs to be sure that the sprayer can discharge different rates and volumes to meet the demands of multiple crops. Knowing how much spray is needed and how long it will take to protect the crops are essential if multiple applications are required and must be deployed within a given time frame. It is also important to recognize that different crops have different spraying needs or volumes, so understanding and planning for appropriate tank volume, portability and hose length should be considered whenever purchasing new equipment.

Sprayer engineering is about trade-offs. Low-volume applications use less water to create small droplets that can result in uniform coverage and greater likelihood of remaining on the target and not running off. However, smaller droplets tend to evaporate quickly – when the humidity is low, smaller droplets may not reach the target, and they are more prone to drift. Conversely, with hydraulic sprayers,

Fig. 9.5. Sprayer components. Sprayers are highly engineered pieces of equipment designed to provide the appropriate dose of a product to the target. (Authors' own figure.)

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spray material is usually applied with less risk of drift, in much greater volumes (190 litres or more), but with a greater risk of runoff and the issue of obtaining large volumes of water for spraying.

Sprayer types

Backpack (or knapsack) sprayers

A backpack (or knapsack) sprayer is effective and efficient for smaller plots (<1 ha) in size, or in mountainous areas. Backpack sprayers are carried on the back (use of a waist strap is recommended to redistribute the weight and reduce strain), and consist of a tank, pump, regulator and at least one nozzle to atomize the pesticide spray (Fig. 9.5). Tank size varies between 10 and 30 litres, limiting their use for high-volume applications. Tank contents may need mixing if they do not have some type of agitator to keep pesticides in suspension.

Most backpack sprayers have a pump (powered by hand or motor) to generate the pressure necessary to drive the pesticide to the target. Diaphragm pumps are easier to maintain and have better durability, but the piston pumps generate greater pressure. Some pumps are capable of high pressures (up to 1200 kPa or 175 psi) but the recommended working pressure is in the range of 100–690 kPa (15–100 psi), enabling coverage of taller plants and trees. The operator controls the application via a trigger valve. These types of sprayers commonly utilize a lance to maintain applicator distance away from the application. Longer lances facilitate the spraying in difficult-to-reach locations. Motorized backpack sprayers can use a two- or four-stroke internal combustion engine or electricity via a battery; pumps can be piston (uniflow), diaphragm or gear to deliver pesticides. These reduce the fatigue caused by pumping but are often heavier. Travel speed, attention to detail, fatigue and many other factors result in variation in application efficiency.

Air blower sprayers

Air blower sprayers use a fan to propel air at high velocity to discharge the pesticide. Coverage is better than that delivered by lance but is more prone to drift. For smaller orchards or nurseries (0.4–2 ha) with trees on dwarf rootstocks, vineyards or coffee plantations, smaller mechanical mist or air blower sprayers provide better canopy penetration and pesticide delivery. Air blowers can be carried on a backpack; when mounted on a vehicle they are called *air blast sprayers* (Fig. 9.6). These can deliver the pesticide into the trees or vines and distribute it throughout the canopy. They are necessary for fungicide to reach the tops of large trees and the canopy interior without the high pressure or heavy streams of liquid with large droplets that run off rather than protect the target. Early in the season, when trees are dormant or just after bud break but before bloom, spraying requires very little air (unless wind is an issue). However, as tree or vine canopies develop and mature, greater volumes of air and pesticide are needed to achieve good coverage (Deveau, 2015). This air volume can be modified by the fan speed or by the tractor/PTO, depending upon how the sprayer is engineered. To correctly deliver the pesticide to the tree or vine, the applicator needs to be aware of the air direction of the sprayer, the nozzle alignment and the pesticide trajectory in relation to canopy. Different nozzle types and sizes may be needed to deliver the most effective application. Air blast applications are very prone to drift and should not be used when wind speeds exceed 8 km/h (5 mph).

Hydraulic sprayers

Hydraulic sprayers utilize a pump that supplies the energy to deliver the spray material to the target plant with water as the carrier. Handgun sprayers are mounted on all-terrain vehicles (ATVs), trucks, tractors, or skid-mounted sprayers with an engine-powered pump. As a result, these sprayers produce larger droplet sizes, 200–400 µm. Low-volume (LV) sprayers generate mist (50–100 µm) or even fog (0.05–50 µm) sized droplets, with water or oil as the carrier. In an LV sprayer, spray material in a water or oil carrier is injected into a high-speed air stream generated by a fan, blower or compressor. In most LV sprayers, a small pump is used to inject a stream of concentrated pesticide solution into the air stream. The speed of the air stream may approach 320 km/h (200 mph).

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Fig. 9.6. An air blast sprayer engineered with 'smart' technology that uses lasers to turn on and off between trees, reducing the pesticide application. (Authors' own photo.)

Compressed air sprayers

Compressed air sprayers consist of a small (4–20 litre) tank, an air pump and a lance with a nozzle. These types of pumps are used for small treatment areas or spot treatments. They require frequent pumping to maintain pressure and have a more variable droplet size due to pressure gain and loss.

Spot sprayers

Spot sprayers are medium-sized sprayers of between 40 and 100 litres. These sprayers are often cart-mounted, making them nimble and versatile, particularly for applications that occur between distant locations. They can be both high- and low-volume sprayers.

Skid-mounted sprayers

Skid-mounted sprayers are mounted on a metal frame which is then bolted to a cart that can be pulled by hand or tractor or mounted on an ATV. Tank sizes can be as large as 750 litres. The pump is powered by a small engine (electric or internal combustion) and often has either a boom with nozzles or a hose reel. This allows sprayers to be removed and vehicles used for multiple purposes.

Boom sprayers

Boom sprayers utilize a concept from sailing, wherein a boom is a pole that improves both the control of the angle and shape of the sail. For pesticide applications, a boom performs the same function, controlling the angle and shape of the pesticide application. This control is achieved via the choice and distribution of nozzles and the distance of the boom from the intended target. Boom length can be 1–3 m for small applications or for vertical use in a vineyard or orchard, or can be as long as 40 m. A boom can be wet or dry: a wet boom describes a pipe that is used as

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both a support mechanism for the spray nozzles and also delivers spray solution; a dry boom is solid and rigged with separate hoses that deliver the pesticide to the nozzle.

When using larger sprayers like boom or air blast, the final volume in the tank should consider any additional volume needed to prime the pump.

Electrostatic sprayers

Electrostatic sprayers exploit the property that all molecules have a charge – positive, negative or neutral. Just as in magnets, opposite charges attract while like charges repel, as per Coulomb's law. In an electrostatic sprayer, the droplets are given a negative charge as they are expelled from the nozzle. These negatively charged droplets are then attracted to the positively charged leaf surface. Due to their negative charge the molecules also repel each other, creating a more uniform coverage. However, studies showing a corresponding improvement in pesticide efficacy, particularly for fungicides, are lacking.

Rotary disc sprayers

A rotary disc sprayer uses a spinning disc to break the application stream into 60–80 μm diameter droplets. When the liquid is metered on to the disc surface, centrifugal forces move the liquid to the edge of the disk, and droplets are produced near the edge. Droplet formation can be controlled by external sources, providing greater control than conventional hydraulic spray nozzles.

Thermal foggers

Thermal foggers use heat to vaporize the fogging solution, forming clouds of thick, white smoke in greenhouse situations. To improve uniformity of droplet size and distribution of the spray material, a carrier is mixed with the pesticide that causes the particles to float in the air for up to 6 h, a disadvantage if personnel have to get into the greenhouse to care for the plants. These sprayers are extremely noisy and additional hearing protection is necessary. Furthermore, few companies have done research regarding the safety and efficacy of their products when used through any type of fogger; recommendations for the use of fungicide delivery via this method are rarely provided on a pesticide label.

Cold (or mechanical) foggers

A cold (or mechanical) fogger uses a high-pressure pump (7–21 MPa or 1000–3000 psi) coupled with atomizing nozzles to produce particles with volume median diameter (VMD) >25 μm that are released into the air stream. The pesticide is distributed via a hand-held lance or external fan unit. Ultra-low-volume formulations are used, and carriers are not needed. This fogger is usually operated by automation, minimizing pesticide exposure to the applicator.

Regardless of the type of sprayer used, all sprayers need to be maintained regularly to ensure effective operation and the uniform delivery of the fungicide to the target crop.

Nozzles

The correct and appropriate use of nozzles is essential to improving droplet-deposition efficiency along with the spatial distribution of droplets throughout the plant canopy, including the often-overlooked leaf underside. Nozzles are essential to this process as they deliver a droplet size that balances coverage, canopy penetration, droplet deposition and adherence to target for effective fungicide use. Ineffective fungicide applications and failure to achieve pesticide deposition on the target can result in >60% loss of efficacy (Law, 2001).

Nozzles are relatively simple piece of equipment. They comprise a pipe or tubing with various inserts that direct and regulate the shape and pressure of liquid sprayed over a given area, controlling both the application rate and spray consistency (Fig. 9.7). The role of the nozzle is to atomize/aerosolize the liquid into droplets of the required size, disperse those droplets into a defined pattern and regulate the liquid at a specific output called the *flow rate*. The flow rate is stated in volume (litres or gallons) per minute. Some pesticide labels specify the allowed volume to be applied to a given area.

Pressure impacts every characteristic of spray performance and for best performance, spray nozzles should be run within the operating parameters recommended by the equipment manufacturer. Nozzles are optimized for different pressures and types of application, but for all

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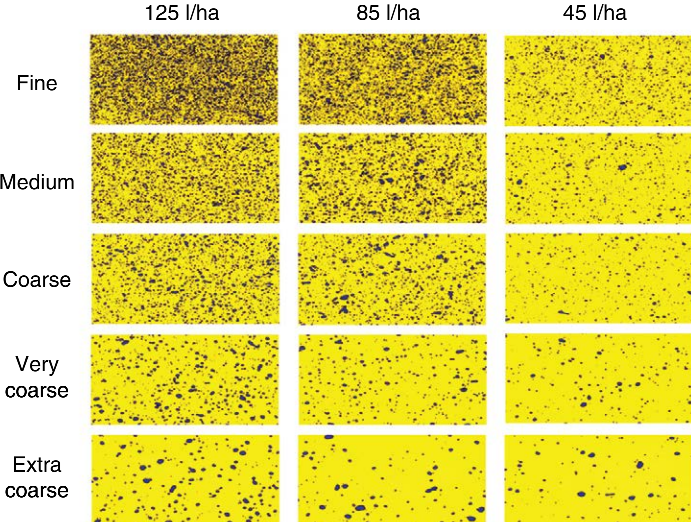

Fig. 9.7. Flow rate and droplet size directly impact target coverage. (From Deveau, 2015, used with permission.)

nozzles, increasing pressure results in: (i) decreasing droplet size; (ii) increasing spray volume; (iii) increasing drift potential; and (iv) loss in nozzle durability. It is important to note that to increase nozzle output, the pressure must be multiplied by the *square* of the desired increase in flow rate; that is, to double the output from a nozzle, the pressure must be quadrupled. Manipulating pressure allows the user to make minor changes to application rate during application, and this is how rate controllers adjust for changes in ground speed while providing a constant rate of application in a given area. However, excessive pressure can result in both suboptimal spray performance and excessive nozzle wear.

For any given application, the choice of nozzle is predicated on how to improve the efficacy of a pesticide while minimizing the risk of drift. In addition to droplet size, spray coverage can be determined by the *spray angle* of the liquid as it exits the nozzle. Nozzles often include components that direct spray output (measured in degrees). The spray angle affects the area of spray coverage at a specific distance, with wider angles treating larger areas (Fig. 9.8). The nozzle and its positioning via height and spacing of the boom also control how uniformly this spray is distributed and can be controlled to reduce drift.

Spray velocity measures the speed of the droplets as they exit the nozzle orifice, and is directly impacted by spray angle, operating pressure and droplet size. Wider spray angles reduce velocity whereas more pressure increases droplet velocity and distance before gravity, turbulence and wind impact their delivery, causing the droplets to fall.

Some labels provide additional information regarding nozzle specification; this is most often provided to minimize the risk of drift (Fig. 9.9). Nozzle choice is determined by the size and pressure needed to obtain the desired volume over time or distance. Confirmation ideally includes performing a trial run to certify that the nozzle performs as expected using water-sensitive paper. Alternatively, using a kaolin clay (like Surround™) will allow for a visual evaluation of spray quality. If the spray is too coarse or too fine a different nozzle should be selected.

Nozzles are the least expensive part on a sprayer and are overlooked at the user's peril, for all the reasons previously listed. Nozzles are the essential tool to produce a droplet that provides appropriate coverage, penetration and deposition, for effective fungicide use (Fig. 9.10). They play the most important job in a pesticide application by fulfilling the maximum efficacy from the fungicide while minimizing drift. Fungicides work best when the appropriate labelled rates are applied during the application, and nozzles are one of the most important components

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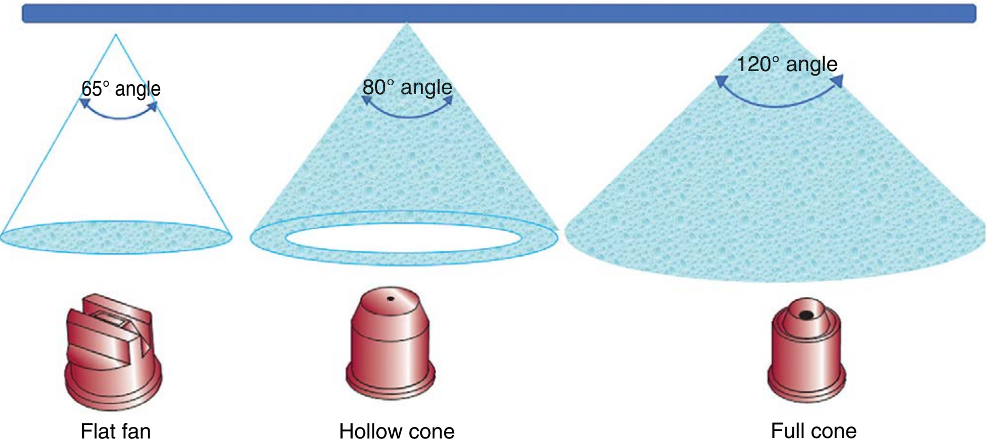

Fig. 9.8. Nozzle type determines distribution pattern and spray angle. (Authors' own figure.)

SPRAY DRIFT MANAGEMENT

Avoiding spray drift at the application site is the responsibility of the applicator. The interaction of many equipment- and weather-related factors determine the potential for spray drift. The applicator and the grower are responsible for considering all these factors when making decisions.

Apply only as a medium or coarser spray (ASAE standard 572) or a volume mean diameter of 300 microns or greater for spinning atomizer nozzles.

Apply only when the wind speed is 2–10 mph at the application site.

Additional requirements for aerial applications:

The boom length must not exceed 75% of the wingspan or 90% of the rotor blade diameter.

Release spray at the lowest height consistent with efficacy and flight safety. Do not relase spray at a height greater than 10 feet above the crop canopy.

When applications are made with a crosswind, the swath will be displaced downwind. The applicator must compensate for this displacement at the downwind edge of the application area by adjusting the path of the aircraft upwind.

Do not make applications into temperature inversions.

Additional requirements for ground boom application:

Do not apply with a nozzle height greater than 4 feet above the crop canopy.

Fig. 9.9. Spray drift management is included in many fungicide labels. (Authors' own figure.)

for ensuring this (Fig. 9.11). Using spray tips that result in a rate below or above what is needed can diminish pesticide efficacy or waste product and provide unnecessary runoff. Using the wrong nozzle can result in a spray capacity or rate that violates the label and the law.

Not surprisingly, different nozzles are available for different uses and different sprayers, and the 'best' nozzle will comply with the label and provide the most efficacy via droplet size while minimizing spray drift. Using only one nozzle would be similar to using only one fungicide and will ultimately compromise the efficacy of certain pesticides, particularly for growers who have a diversity of crops (e.g. vegetable and ornamental growers) and/or pathogens to manage (Fig. 9.12). A nozzle that provides fine droplets will perform well against foliar diseases, but the same nozzle would not provide an application to reach the crown of the plant to protect against *Rhizoctonia*, *Sclerotinia* or a diversity of crown rots if applied as a drench.

When selecting the appropriate nozzle, the following should be considered:

- the target (field crops, orchard and vineyard, nursery, greenhouse, landscape, vegetables)

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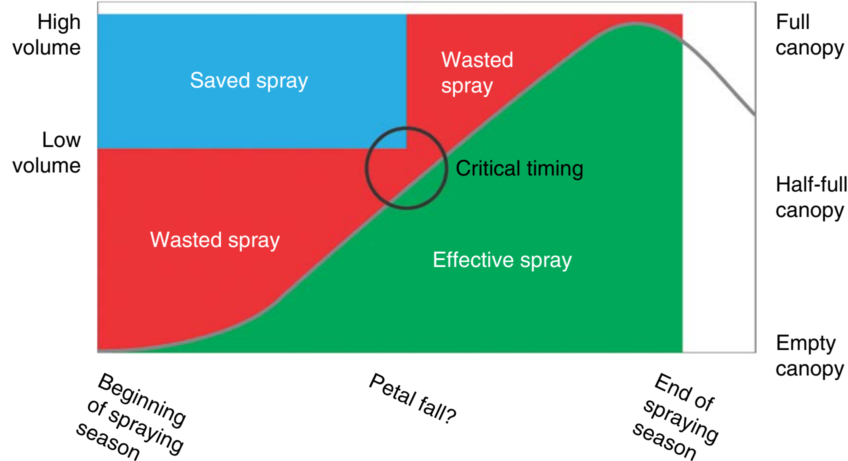

Fig. 9.10. By changing nozzles, volumes and/or travel speed over time to coincide with canopy development, fungicide inputs can be reduced (blue) without compromising coverage or protection. (Image used with permission from Deveau, 2021.)

Fig. 9.11. Multiple nozzle bodies on a turret facilitates nozzle change. (Used with permission by TeeJet Technologies.)

and the appropriate level of coverage for the fungicide applied (protectant or systemic);

- the risk of drift;
- the application rate (considering spray pressure and speed of equipment);
- the application type (air-assisted, broadcast, banded, direct, drench);
- the spray pressure and travel speed requirements; and
- sprayer operation parameters (include application rate, pressure, speed, and if any borders are required to minimize drift or groundwater contamination).

Liquids can be transformed into droplets and aerosolized by nozzles that are engineered to deliver pesticide applications by breaking and dispersing droplets in a specific pattern while providing uniformity and meeting specifications. Agricultural nozzles accomplish this by utilizing air shear, centrifugal energy and hydraulic pressure. Hydraulic nozzles utilize the energy of the fluid stream (pressure) to shatter the flow into the pattern of interest. The conventional hydraulic nozzle is a device with a feed line that leads to a smaller orifice. When liquid is forced through the nozzle tip under pressure, the resulting hydraulic energy destabilizes the liquid, disintegrating it into spray droplets. This disintegration process is largely uncontrolled, resulting in a wide range of droplet sizes. Most large-scale fungicide applications use hydraulic nozzles. Droplet size increases with the use of hollow cone, followed by flat fan and full cone nozzles.

At a certain distance from the nozzle tip, nozzle patterns degrade to a mist or fog, regardless of whether it is a full or hollow cone. Some nozzles are deliberately designed to produce a

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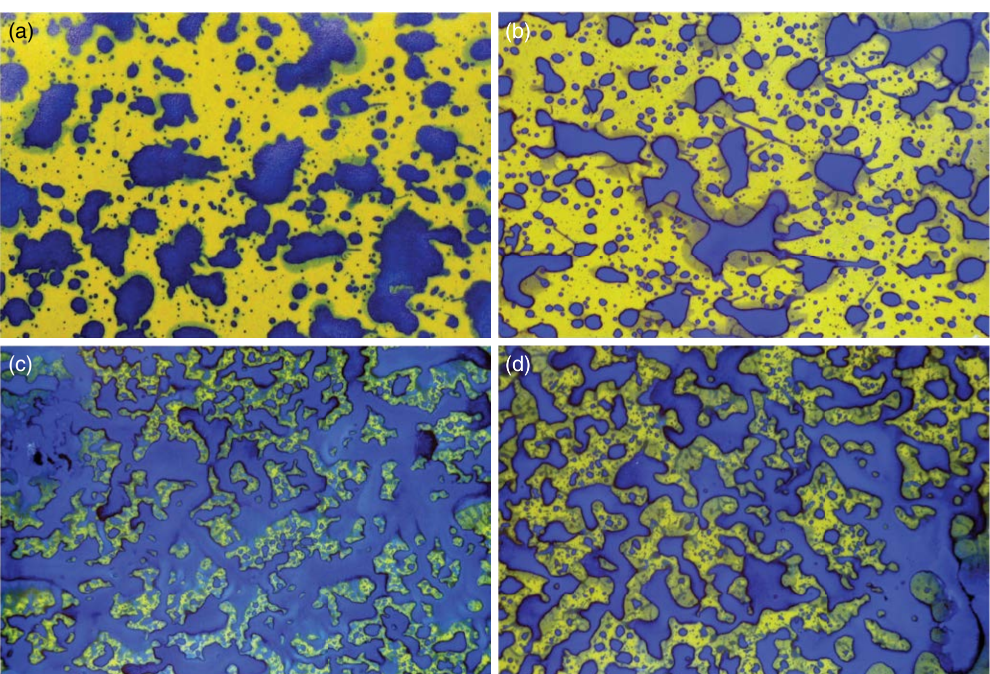

Fig. 9.12. Yellow moisture-sensitive paper sprayed with four different nozzles at 50 gal/acre (1 gal/acre = 9.3540 l/ha): (a) extremely coarse droplet (raindrop); (b) extremely coarse droplet (TurfJet nozzle); (c) medium droplet (XR TeeJet nozzle); (d) air-induction, very coarse droplet. The sprayed areas are blue, and the dry areas remain yellow. Nozzles that produce smaller droplets improve coverage. (From Shepard *et al*., 2006, used with permission.)

mist or fog. The distinction between mist or fog is defined by droplet size, with mists having larger droplet sizes and fogs having much smaller ones. Not surprisingly, a hydraulic nozzle will produce finer droplets resulting in fog as pressure is raised; mist patterns have a lower flow rate with larger droplet sizes and little impact. As such, designations of fog nozzle or misting nozzle are used interchangeably, just as the nozzles can be, depending upon pressure. For agricultural uses, these nozzles are usually limited to greenhouses and enclosed places.

Fan nozzles

FLAT-FAN NOZZLES. The elliptical opening of a flat-fan nozzle emits a flattened conical pattern that can have a narrow or wide angle (Fig. 9.8). Regardless of angle, sprayer output is focused in the centre of the pattern, decreasing towards the outer edge. For effective coverage with flat-fan nozzles, a 30–50% overlap between the nozzles in needed; ultimately, though, the spacing of nozzles along with boom height determine the degree of spray overlap. If pressure is decreased, overlap of output may not occur and result in poor coverage. Flat-fan nozzles are commonly used for broadcast spraying and produce coarser droplets, along with greater penetration into the plant canopy. Newer extended-range nozzles are capable of operating from 100 to 410 kPa (15 to 60 psi) without impinging on the width of the spray pattern. One variation of the flat fan, the even flat fan, produces a consistent spray that does not require overlap.

DEFLECTION FLAT-FAN NOZZLES. The deflection flat-fan nozzle uses a circular orifice to produce a flat-fan pattern but deflects the pesticide stream against a curved surface after exit from the nozzle orifice, creating the flat fan and desired droplet size. The benefit of this type of nozzle is less wear than the elliptical one. This type of nozzle is most often used for boom sprayers to apply pesticides to the upper portion of the plant.

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DUAL FLAT-FAN NOZZLES. Dual flat-fan nozzles have two fan-like jets set at an angle providing dual flat spray simultaneously to two different angles compared with a single fan, which is only vertical. This provides a spray that is facing both the 'front' and the 'back' of the plant and is particularly important for vertical or upright crops like wheat, vertical vegetable crops like asparagus, celery and onions, or any other crop where the exposed vertical part of the plant canopy is the primary spray target (e.g. Fusarium head blight, wheat blast, smuts). Dual nozzles use higher flow-rate nozzles which reduces droplet size, improving coverage.

FLOODING (FLAT-FAN) NOZZLES. Flooding (flat-fan) nozzles produce a wide-angle flat pattern with large spray droplets. These nozzles are commonly used for drenches or fertilizer–pesticide tank-mixes. Nozzles need to provide overlap for complete coverage. These nozzles are often used for applying liquid fertilizers or fertilizer–pesticide mixtures or for directing herbicide sprays under plant canopies.

PNEUMATIC/AIR-ACTUATED FLAT FANS. Pneumatic/air-actuated flat fans use the on–off cycling (multiple times per second) of the nozzle to produce a coarse-to-fine atomized spray. This level of control is especially important in 'smart sprayers' in precision agriculture, that deploys sensors and lasers to identify targets and only applies fungicide to the target, as opposed to an area that contains the target.

Cone nozzles

These can be hollow cone or solid cone design. Both types are commonly used in fungicide and insecticide applications that require both penetration and coverage of plant foliage.

HOLLOW CONE NOZZLES. A hollow cone spray nozzle forms a ring pattern that results from the heavy deposition of droplets around the edge of the spray cone and little to no pesticide deposited in the middle of the cone, thus the hollow (Fig. 9.8). This hollow cone forms when the nozzle swirls the pesticide by an internal vein, core or whirl plate or whirl chamber, forcing the liquid to rotate or swirl. The swirling creates turbulence that breaks the liquid into droplets which are then shaped into a hollow cone pattern as they exit the orifice. This pattern results in less impact (and shatter and runoff) than flat fan or solid stream patterns, but greater impact than full cone patterns. This design of this nozzle can also be used to produce a full cone pattern under low pressure.

For the hollow cone nozzle, the spray pattern can be manipulated by the geometry of the whirl chamber and how the pesticide exits the orifice: tangential whirl nozzles provide the widest spray trajectory and resistance to clogging; narrow or axial whirl nozzles create a tight pattern; and spiral whirl nozzles have a broad range of spray angles, but often poor distribution of output.

Hollow cone nozzles are often used to apply fungicides to field crops, and whenever small droplets with fine spray coverage is desired. Cone-type nozzles are commonly used in orchards and vineyards for fruit crops under a high-pressure application, like air blast, to provide penetration and reach both inner leaves and their undersides and provide greater deposition into the canopy. To do this at higher pressure, many hollow cone nozzles use a disc and core (Fig. 9.4) to facilitate spraying pesticides at higher pressures and flow rates and at different angles; larger-capacity nozzles are often used in air blast sprayers which regularly operate at 1375–2070 kPa (200–300 psi). Both discs and cores are available in a variety of sizes and materials and include ceramic, hardened stainless steel, stainless steel, plastics and/or nylon polymers. These nozzles work especially well with wettable powders and other abrasive chemicals, like mancozeb.

FULL CONE NOZZLES. The full or solid cone nozzle delivers a full, round pattern with even coverage (Fig. 9.8). Within the full cone nozzle, the fluid is swirled by a whirl chamber or internal vein. Turbulence aerosolizes the liquid into droplets which are then shaped into a full cone pattern upon exiting the orifice. The full cone pattern is maintained for a specific distance – any increasing distance results in mist or fog that can drift off site. Just like the hollow cone, four basic designs of nozzle can produce a full cone pattern: full cone axial whirl, tangential whirl, spiral whirl and full cone air atomizing.

As the name suggests, whirl nozzles force the pesticide stream to be whirled as it leaves the

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nozzle and come in two forms. In an *axial full cone*, the pesticide is spun against a central axis, internal vein or whirl chamber (hence, axial). For a *tangential full cone*, the spray pattern is formed by the fluid as it enters the nozzle body tangentially and is twisted through 90° in a whirl motion out the side. This causes it to break up into droplets before exiting the spray orifice. The addition of a contoured insert directs fluid to the centre, forming a full cone pattern instead of a hollow cone. This design is more resistant to clogging than axial whirls as the whirl chamber is less intricate.

The use of a spiral/helical cone nozzle results in impact of the liquid on to the protruding helix upon exit from the spray orifice. The liquid breaks into droplets, forming the spray pattern as it shears off the helix. The spray pattern consists of concentric rings composed of multiple hollow cone spray patterns. This results in less uniformity of liquid distribution leading to an 'approximate' full cone pattern. Changes in helix geometry can result in a hollow cone pattern. Regardless of flow rate or pressure, spiral nozzles produce what is a full cone spray with the smallest droplet size for any direct pressure nozzles, but because the spray pattern consists of overlapping hollow cones, coverage is not as uniform as with other nozzles. Although there is a loss of uniformity, the heavier droplets in the overlapping rings 'pull' the smaller, lighter droplets along with the spray, providing improved canopy penetration, and deliver smaller droplets where they would not otherwise reach. Lastly, the spiral whirl is more resistant to clogging, which allows different formulations of pesticides (WP, WDG, EC, SC, etc.) to be sprayed. These nozzles are less prone to blockage as they have larger free passages.

Other nozzle types

IMPINGEMENT NOZZLES. Another type of misting nozzle is called an impingement nozzle. This nozzle possesses a pin at the tip and functions like other nozzles in that fluid is ejected via a small orifice and atomized upon impact by the pin. These nozzles are prone to clogging. Their use is usually limited to greenhouse operations.

CONTROLLED DROPLET APPLICATORS. Controlled droplet applicators (CDAs), also called *rotary nozzles* or centrifugal energy atomizers, create a narrower range of droplet sizes compared with other nozzles. Centrifugal force propels liquid through a spinning disc or rotating mesh cage that produces ligaments; droplet size is inversely proportional to rotor speed, with faster speeds resulting in smaller droplets. CDAs prove most useful in areas where water is scarce, particularly in developing countries. Sprayers can be hand-held or mounted on a tractor.

ELECTROSTATIC NOZZLES. Electrostatic nozzles are a newer technology that may result in reduced rates of fungicide for comparable control – to date, most studies are inconsistent. Spray is charged (positive or negative) by a cathode or an electrode positioned within the spray output where drops begin formation. As previously stated, many factors affect droplet contact with the target, and electrostatic charge would require that the droplet be in close vicinity to the target for benefits to be realized. Many of these droplets are smaller which, if able to hit the target, could result in better control with less pesticide. However, this would more likely be true in a greenhouse or other enclosed environment, rather than a field application.

AIR SHEAR NOZZLES. Air shear nozzles are different from the previously mentioned nozzles as they use high-speed discharge to break up and aerosolize liquid. These nozzles are limited to air blast sprayers that can provide the discharge necessary to propel the fungicide. When the liquid is ejected into the air stream against the flow, it becomes sheared, producing droplets. This process allows for wide orifices and minimizes plugging and nozzle wear. The droplet size is controlled by the ratio of liquid to air (i.e. reducing liquid flow with increasing air velocities produces smaller droplets).

How to choose a nozzle

Nozzle selection appears to be overwhelming, but choices are often limited by label restrictions, the sprayer, desired sprayer output and field speed (Table 9.1). Nozzle selection information is provided on manufacturers' websites, smartphone apps or charts. Nozzle manufacturers include all the factors that must be considered to choose the appropriate nozzles and include tractor speed, spray volume and the pressure used.

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Table 9.1. Droplet size categories (ASABE) are classified by the volume median diameter (VMD) of a range of droplet sizes. Colour-coded tables identify the VMD droplet size for different nozzles and at various pressures. Droplet size classification by nozzle type, pressure, droplet spectra classification and flow rate provide consistency for applicators. (Used with permission by TeeJet Technologies.)

TeeJet TECHNOLOGIES

Droplet Size Data Based on ISO 25358 Standard

Nozzle	PSI	110°XR/XRC 15-60 PSI	TT 15-90 PSI	TTJ60 20-90 PSI	AIXR 15-90 PSI	AI3070 20-90 PSI	AITTJ60 20-90 PSI	110°AI/AIC 30-115 PSI	TTI60 20-90 PSI	TTI 15-100 PSI	GPM	GPA 20" 4 mph	5 mph	6 mph	7 mph	8 mph	9 mph	10 mph	12 mph	14 mph	16 mph	18 mph	20 mph
015 AI AIC AIXR AI3070 TT TTI XR XRC (100)	20	F	VC	—	VC	XC	—	—	—	UC	0.11	8.2	6.5	5.4	4.7	4.1	3.6	3.3	2.7	2.3	2.0	1.8	1.6
	30	F	C	—	C	VC	—	XC	—	UC	0.13	9.7	7.7	6.4	5.5	4.8	4.3	3.9	3.2	2.8	2.4	2.1	1.9
	40	F	M	—	C	VC	—	XC	—	XC	0.15	11.1	8.9	7.4	6.4	5.6	5.0	4.5	3.7	3.2	2.8	2.5	2.2
	50	F	M	—	M	C	—	VC	—	XC	0.17	12.6	10.1	8.4	7.2	6.3	5.6	5.0	4.2	3.6	3.2	2.8	2.5
	60	F	M	—	M	C	—	VC	—	XC	0.18	13.4	10.7	8.9	7.6	6.7	5.9	5.3	4.5	3.8	3.3	3.0	2.7
	70	—	M	—	M	M	—	VC	—	VC	0.20	14.9	11.9	9.9	8.5	7.4	6.6	5.9	5.0	4.2	3.7	3.3	3.0
	80	—	F	—	M	M	—	C	—	VC	0.21	15.6	12.5	10.4	8.9	7.8	6.9	6.2	5.2	4.5	3.9	3.5	3.1
	90	—	F	—	M	M	—	C	—	VC	0.23	17.1	13.7	11.4	9.8	8.5	7.6	6.8	5.7	4.9	4.3	3.8	3.4
02 AI AIC AIXR TT TTI TTI60 XR XRC (50) AI3070 AITTJ60 TTJ60 (100)	20	M	VC	C	VC	XC	XC	—	UC	UC	0.14	10.4	8.3	6.9	5.9	5.2	4.6	4.2	3.5	3.0	2.6	2.3	2.1
	30	F	C	C	VC	VC	VC	XC	XC	UC	0.17	12.6	10.1	8.4	7.2	6.3	5.6	5.0	4.2	3.6	3.2	2.8	2.5
	40	F	M	M	C	VC	VC	XC	XC	XC	0.20	14.9	11.9	9.9	8.5	7.4	6.6	5.9	5.0	4.2	3.7	3.3	3.0
	50	F	M	M	M	C	C	VC	VC	XC	0.22	16.3	13.1	10.9	9.3	8.2	7.3	6.5	5.4	4.7	4.1	3.6	3.3
	60	F	M	M	M	C	C	VC	VC	XC	0.24	17.8	14.3	11.9	10.2	8.9	7.9	7.1	5.9	5.1	4.5	4.0	3.6
	70	—	M	M	M	M	C	VC	VC	VC	0.26	19.3	15.4	12.9	11.0	9.7	8.6	7.7	6.4	5.5	4.8	4.3	3.9
	80	—	F	M	M	M	C	C	C	VC	0.28	21	16.6	13.9	11.9	10.4	9.2	8.3	6.9	5.9	5.2	4.6	4.2
	90	—	F	M	M	M	M	C	C	VC	0.30	22	17.8	14.9	12.7	11.1	9.9	8.9	7.4	6.4	5.6	5.0	4.5
025 AI AIC AIXR TT TTI TTI60 XR XRC (50) AI3070 AITTJ60 TTJ60 (100)	20	M	VC	VC	VC	XC	XC	—	UC	UC	0.18	13.4	10.7	8.9	7.6	6.7	5.9	5.3	4.5	3.8	3.3	3.0	2.7
	30	M	C	C	VC	VC	VC	XC	XC	UC	0.22	16.3	13.1	10.9	9.3	8.2	7.3	6.5	5.4	4.7	4.1	3.6	3.3
	40	F	M	M	C	VC	VC	XC	XC	XC	0.25	18.6	14.9	12.4	10.6	9.3	8.3	7.4	6.2	5.3	4.6	4.1	3.7
	50	F	M	M	M	C	VC	VC	VC	XC	0.28	21	16.6	13.9	11.9	10.4	9.2	8.3	6.9	5.9	5.2	4.6	4.2
	60	F	M	M	M	C	C	VC	VC	XC	0.31	23	18.4	15.3	13.2	11.5	10.2	9.2	7.7	6.6	5.8	5.1	4.6
	70	—	M	M	M	C	C	VC	VC	VC	0.33	25	19.6	16.3	14.0	12.3	10.9	9.8	8.2	7.0	6.1	5.4	4.9
	80	—	F	M	M	M	C	C	C	VC	0.35	26	21	17.3	14.9	13.0	11.6	10.4	8.7	7.4	6.5	5.8	5.2
	90	—	F	M	M	M	C	C	C	VC	0.38	28	23	18.8	16.1	14.1	12.5	11.3	9.4	8.1	7.1	6.3	5.6
03 AI AIC AIXR AITTJ60 AI3070 TT TTI TTI60 XR XRC (50) TTJ60 (100)	20	M	VC	VC	VC	XC	XC	—	UC	UC	0.21	15.6	12.5	10.4	8.9	7.8	6.9	6.2	5.2	4.5	3.9	3.5	3.1
	30	M	C	C	VC	VC	XC	XC	XC	UC	0.26	19.3	15.4	12.9	11.0	9.7	8.6	7.7	6.4	5.5	4.8	4.3	3.9
	40	F	M	C	C	VC	VC	XC	XC	XC	0.30	22	17.8	14.9	12.7	11.1	9.9	8.9	7.4	6.4	5.6	5.0	4.5
	50	F	M	M	C	VC	VC	VC	XC	XC	0.34	25	20	16.8	14.4	12.6	11.2	10.1	8.4	7.2	6.3	5.6	5.0
	60	F	M	M	M	C	C	VC	XC	XC	0.37	27	22	18.3	15.7	13.7	12.2	11.0	9.2	7.8	6.9	6.1	5.5
	70	—	M	M	M	C	C	VC	VC	VC	0.40	30	24	19.8	17.0	14.9	13.2	11.9	9.9	8.5	7.4	6.6	5.9
	80	—	F	M	M	C	C	C	VC	VC	0.42	31	25	21	17.8	15.6	13.9	12.5	10.4	8.9	7.8	6.9	6.2
	90	—	F	M	M	M	C	C	VC	VC	0.45	33	27	22	19.1	16.7	14.9	13.4	11.1	9.5	8.4	7.4	6.7
04 AI AIC AITTJ60 AIXR AI3070 TT TTI TTI60 TTJ60 XR XRC (50)	20	M	VC	VC	VC	XC	XC	—	UC	UC	0.28	21	16.6	13.9	11.9	10.4	9.2	8.3	6.9	5.9	5.2	4.6	4.2
	30	M	C	C	VC	XC	XC	XC	UC	UC	0.35	26	21	17.3	14.9	13.0	11.6	10.4	8.7	7.4	6.5	5.8	5.2
	40	M	M	C	C	VC	VC	XC	XC	XC	0.40	30	24	19.8	17.0	14.9	13.2	11.9	9.9	8.5	7.4	6.6	5.9
	50	F	M	M	C	VC	VC	VC	XC	XC	0.45	33	27	22	19.1	16.7	14.9	13.4	11.1	9.5	8.4	7.4	6.7
	60	F	M	M	C	C	C	VC	XC	XC	0.49	36	29	24	21	18.2	16.2	14.6	12.1	10.4	9.1	8.1	7.3
	70	—	M	M	M	C	C	VC	VC	VC	0.53	39	31	26	22	19.7	17.5	15.7	13.1	11.2	9.8	8.7	7.9
	80	—	F	M	M	C	C	C	VC	VC	0.57	42	34	28	24	21	18.8	16.9	14.1	12.1	10.6	9.4	8.5
	90	—	F	M	M	C	C	C	VC	VC	0.60	45	36	30	25	22	19.8	17.8	14.9	12.7	11.1	9.9	8.9
05 AI AIC AITTJ60 AIXR AI3070 TT TTI TTI60 TTJ60 XR XRC (50)	20	M	VC	VC	XC	UC	UC	—	UC	UC	0.35	26	21	17.3	14.9	13.0	11.6	10.4	8.7	7.4	6.5	5.8	5.2
	30	M	C	C	VC	XC	XC	XC	UC	UC	0.43	32	26	21	18.2	16.0	14.2	12.8	10.6	9.1	8.0	7.1	6.4
	40	M	M	C	VC	VC	VC	XC	XC	XC	0.50	37	30	25	21	18.6	16.5	14.9	12.4	10.6	9.3	8.3	7.4
	50	F	M	M	C	VC	VC	VC	XC	XC	0.56	42	33	28	24	21	18.5	16.6	13.9	11.9	10.4	9.2	8.3
	60	F	M	M	C	VC	C	VC	XC	XC	0.61	45	36	30	26	23	20	18.1	15.1	12.9	11.3	10.1	9.1
	70	—	M	M	C	C	C	VC	VC	VC	0.66	49	39	33	28	25	22	19.6	16.3	14.0	12.3	10.9	9.8
	80	—	F	M	M	C	C	VC	VC	VC	0.71	53	42	35	30	26	23	21	17.6	15.1	13.2	11.7	10.5
	90	—	F	M	M	C	C	C	VC	VC	0.75	56	45	37	32	28	25	22	18.6	15.9	13.9	12.4	11.1
06 AI AIC AITTJ60 AIXR TT TTI TTI60 TTJ60 XR XRC (50)	20	M	VC	VC	XC	—	UC	—	UC	UC	0.42	31	25	21	17.8	15.6	13.9	12.5	10.4	8.9	7.8	6.9	6.2
	30	M	C	C	VC	—	XC	XC	UC	UC	0.52	39	31	26	22	19.3	17.2	15.4	12.9	11.0	9.7	8.6	7.7
	40	M	M	C	VC	—	VC	XC	XC	XC	0.60	45	36	30	25	22	19.8	17.8	14.9	12.7	11.1	9.9	8.9
	50	M	M	M	VC	—	VC	VC	XC	XC	0.67	50	40	33	28	25	22	19.9	16.6	14.2	12.4	11.1	9.9
	60	M	M	M	C	—	VC	VC	XC	VC	0.73	54	43	36	31	27	24	22	18.1	15.5	13.6	12.0	10.8
	70	—	M	M	C	—	C	VC	VC	VC	0.79	59	47	39	34	29	26	23	19.6	16.8	14.7	13.0	11.7
	80	—	F	M	C	—	C	VC	VC	VC	0.85	63	50	42	36	32	28	25	21	18.0	15.8	14.0	12.6
	90	—	F	M	C	—	C	C	VC	VC	0.90	67	53	45	38	33	30	27	22	19.1	16.7	14.9	13.4
08 AI AIC AITTJ60 AIXR TT TTI TTI60 TTJ60 XR XRC (50)	20	M	VC	VC	XC	—	UC	—	UC	UC	0.57	42	34	28	24	21	18.8	16.9	14.1	12.1	10.6	9.4	8.5
	30	M	VC	C	XC	—	XC	XC	UC	UC	0.69	51	41	34	29	26	23	20	17.1	14.6	12.8	11.4	10.2
	40	M	M	C	VC	—	XC	XC	XC	XC	0.80	59	48	40	34	30	26	24	19.8	17.0	14.9	13.2	11.9
	50	M	M	M	VC	—	XC	XC	XC	XC	0.89	66	53	44	38	33	29	26	22	18.9	16.5	14.7	13.2
	60	M	M	M	VC	—	VC	VC	XC	VC	0.98	73	58	49	42	36	32	29	24	21	18.2	16.2	14.6
	70	—	M	M	C	—	VC	VC	VC	VC	1.06	79	63	52	45	39	35	31	26	22	19.7	17.5	15.7
	80	—	M	M	C	—	VC	VC	VC	VC	1.13	84	67	56	48	42	37	34	28	24	21	18.6	16.8
	90	—	F	M	C	—	VC	VC	VC	VC	1.20	89	71	59	51	45	40	36	30	25	22	19.8	17.8
10 AITTJ60 TTJ60 (50) AIC AIXR TT TTI XR XRC	20	C	XC	VC	UC	—	UC	—	—	UC	0.71	53	42	35	30	26	23	21	18	15	13	12	11
	30	C	VC	C	XC	—	XC	UC	—	UC	0.87	65	52	43	37	32	29	26	22	18	16	14	13
	40	M	VC	C	VC	—	XC	XC	—	XC	1.00	74	59	50	42	37	33	30	25	21	19	17	15
	50	M	C	C	VC	—	XC	XC	—	XC	1.12	83	67	55	48	42	37	33	28	24	21	18	17
	60	M	C	M	VC	—	VC	XC	—	VC	1.22	91	72	60	52	45	40	36	30	26	23	20	18
	70	—	C	M	VC	—	VC	VC	—	VC	1.32	98	78	65	56	49	44	39	33	28	25	22	20
	80	—	M	M	C	—	VC	VC	—	VC	1.41	105	84	70	60	52	47	42	35	30	26	23	21
	90	—	M	M	C	—	VC	VC	—	VC	1.50	111	89	74	64	56	50	45	37	32	28	25	22

EXTREMELY FINE (XF) | VERY FINE (VF) | FINE (F) | MEDIUM (M) | COARSE (C) | VERY COARSE (VC) | EXTREMELY COARSE (XC) | ULTRA COARSE (UC)

Spray angle	Height (20")	Tip Spacing	GPA Conversion Factor
80°	30"	15"	1.33
110°	20"	30"	0.67

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Nozzle wear should be monitored routinely because even slight nozzle wear can negatively affect coverage, regardless of nozzle type. Nozzles have a finite life span, and it is essential that they be checked regularly to detect wear. Newer materials last longer than the older brass nozzles, but a nozzle that is worn by only 10% can negatively affect coverage. Nozzles can be evaluated regularly by comparing the flow rate of a used and new nozzle of the same size and type. This can be done easily by using a graduated container, a timing device and a pressure gauge mounted at the nozzle. If the flow from the used nozzle is 10% greater or more, it should be replaced.

The use of water-sensitive paper attached to representative leaves throughout the plant canopy assists in monitoring the coverage and should be used both in calibration and in actual pesticide applications (Fig. 9.7). Spray droplet patterns and density identify the droplet size and distribution (number of droplets that were received by the leaf). Adjustments should be made in subsequent applications to try to improve the results.

Drift prevention

Drift is the term used to describe the movement of pesticide sprays away from the intended crop and on to neighbouring areas. There is a complex interaction between the size of the droplet, the obstacles in its path and the droplet's relative velocity needed to reach the target. When applying pesticides, the inevitable small droplets are easily moved off the target area by the wind. For this reason, the climatic conditions need to be considered. Wind is the primary factor impacting drift and applying pesticides under still conditions is essential. Pesticide labels contain recommendations to account for maximum wind speeds. Wind is not the only factor; higher temperature and lower relative humidity drive evaporation, reducing droplet size and increasing the potential for drift.

To manage drift, the applicator must factor in:

- Droplet size and spray pressure – smaller droplets are produced when smaller nozzle sizes are used with greater spray pressures, resulting in drift in the presence of wind.
- Increasing droplet size or pressure reduces the possibility of drift.
- Volumes – use high output volumes when possible, recognizing label restrictions.
- Tractor/sprayer operating speed – faster speeds can create a wake behind the tractor or sprayer. This can cause the spray to be diverted and redirected away from the target.
- Spray tip height – any increase in distance between the nozzle tip and the target creates greater likelihood that wind could intercept the spray and cause drift.

With the increasing concerns about pesticides, the need to reduce pesticide pollution from drift and runoff has increased. Sprayer and nozzle technology will be important partners in optimizing applications while reducing drift.

Droplets

Fungicide efficacy is impacted by the deposition and persistence of the fungicide applied. Most fungicides are applied as liquid sprays that are atomized and dispersed over the target in the form of *droplets*. Droplets can be formed and manipulated in many ways, including air shear, centrifugal energy, electrodynamic methods and hydraulic pressure, along with kinetic and thermal energy, although hydraulic and centrifugal energy are the most common mechanisms. Different sprayers influence application efficacy by changing any of three droplet properties: droplet size, droplet number and droplet velocity. Although this chapter focuses on droplets, in reality, it is a *deposited residue* that remains on a plant after the pesticide has been delivered. Unlike the deposit, the droplet is a variable we can manipulate, even though the chemical that remains, the deposit, is what provides the efficacy and interesting biological questions.

When considering fungicide applications, it is important to keep in mind the issue of *dose*, which is defined as the quantity of AI necessary to inhibit infection and the amount of chemical deposited per unit area of target. This is distinct from *rate*, which is defined as a fixed ratio between pesticide (formulation + AI) per volume administered to a fixed area. The concept of rate is both useful and necessary for the applicator, but it is not an accurate measure for the scientific

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understanding of biological effects and often confounds discussions regarding pesticide application efficiency. The rate of chemical applied per hectare or acre, or per set volume of water, does not result in nor automatically determine the dose. Dose is a function of the application rate, the coverage and the canopy being sprayed. If droplet size is consistent (from a CDA, for example) and density is known, the dose of chemical reaching the target can be calculated. This is rarely the case for hydraulic nozzles, where the variation of droplet sizes produced makes it difficult to estimate dose: there is a million-fold difference between the dose in a 5 μm and a 500 μm droplet (100^3).

The total applied dose is a function of the number of droplets on the leaf surface, the size of the droplets and the concentration of the AI per droplet. The rate of fungicide applied is always greater than the dose needed, due to the probability that any given spore coming into contact (inoculation) with a susceptible host is a function of the ratio of protected to unprotected plant tissue. Fungicide failure can still result due to poor coverage with a high dose or an excellent coverage with a sublethal one. The most obvious way to control dose and density is via the droplet.

The dose is primarily delivered via droplets. Droplets are formed when the stream or sheet of liquid is ejected from the nozzle in the form of small ligaments that disintegrate into droplets and mist. Primary factors impacting dispersion and deposition include sprayer type, nozzle type, pressure, water volume and water quality (pH, alkalinity, hardness and salts).

Spray exists as distributions of different drop sizes (Fig. 9.12). The Droplet Size Classification Standard S572.1 has been categorized by the American Society of Agricultural and Biological Engineers (ASABE) and the British Crop Protection Council (BCPC). This standard defines and codifies conditions for spray droplet measurement and uses reference nozzle sets to normalize the data. There are several methods to determine droplet size, but most sprayers and nozzles in agriculture use the VMD, a midpoint droplet size where 50% of the spray volume is in droplets smaller than, and the other 50% of spray droplets are larger than, the median. Not surprisingly, different methods exist for spray analysis and produce different results depending upon spray type. The value of the ASABE standard is it that allows comparison between nozzles, thereby controlling differences in both statistical data and interpretation by different measuring equipment to develop a system that provides VMD (Table 9.1; Box 9.3). It is important to realize that a spray nozzle produces a range of droplet sizes, and that this range is often simplified to a single number. The range of droplet sizes plays an important role in completing the coverage of a target: larger droplets provide canopy penetration whereas smaller droplets better adhere to stems, petioles and peduncles that larger droplets fail to adhere to. While the goal is coverage, the relationship between coverage and efficacy is impacted by many other factors.

Within a spray, there will be a range or spread of droplet sizes and accounting for the variability is necessary. The *relative span* (Rs) is a measure of how varied the droplet sizes are in each spray and is defined as:

$$Rs = (DV0.9 – DV0.1)/DV0.5$$

Comparably sized droplets perform similarly, and with similar trajectories, whereas a large relative span in droplet sizes may result in grouping and an inconsistent droplet distribution. Furthermore, larger droplet sizes may indicate the coalescing of smaller droplets; conversely, smaller droplets may indicate the shattering of larger droplets, all of which have implications in what is being measured.

The fate of a pesticide application and its biological efficacy are constrained by several factors and include the complex and competing interactions between droplet size, droplet density, volume of application, concentration of the pesticide and surface of the target. *Retention* is defined as the overall capture of spray droplets and determines the amount of AI on a plant. In fact, the probability of droplet retention by a plant is inversely correlated to droplet VMD, droplet velocity, volume of application, plant surface (dynamic contact angle), plant structure and plant canopy density. The data to optimize these aspects of pesticide application are severely lacking for fungicide efficacy in most crops, with a fraction of the studies examining these crucial interactions in pesticide efficiency compared with insecticide and herbicide studies, representing an often-overlooked opportunity to improve pesticide performance.

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Box 9.3. Measuring droplet size.

A spray nozzle produces a range of droplet sizes that can often be summarized by a single number. Which single number is appropriate when comparing the droplet spectrum of several sprays depends upon the application and what is being measured.

When measuring droplet size within a spray, it should be recognized that different methods for analysing sprays will produce different results depending on the type of spray. It is important to understand which measurement system is being used, and particularly to ensure that the same method is used when comparing the sprays produced by two different nozzles.

These different measurements include:

- Arithmetic mean diameter (D10) – the average of the diameters of all the droplets in the spray sample.
- Sauter mean diameter (D32) – the diameter of a droplet whose ratio of volume to surface area is equal to that of the complete spray sample.
- Volume median diameter (D30) – the diameter of a droplet whose volume, if multiplied by the total number of droplets, will equal the total volume of the sample.
- Mass (volume) median diameter (DV0.5) – the diameter which divides the mass (or volume) of the spray into two equal halves. Thus half of the total mass is made up of droplets with diameters smaller than this number and the other half with diameters that are larger. This measurement is more commonly used when the drift of a fluid is important.
  - DV0.1 would represent the diameter that 10% of droplets are smaller than and DV0.9 would represent the diameter that 90% of droplets are smaller than.

When evaluating these numbers and comparing droplet size distributions, the Sauter mean diameter, D32, is larger than the arithmetic, D10, surface, D20, and volume, D30, mean diameters.

As previously stated, pesticide applications are about trade-offs. Larger size droplets are more successful in penetrating the canopy and less likely to drift than smaller droplets. The use of larger droplets provides greater opportunity to apply pesticides, as smaller droplets may drift during windier conditions. Larger droplets can lead to greater deposition on the host plant: upon impact of the larger drops and droplets, shattering and bounce increase the probability of contact with the target (Zabkiewicz *et al.*, 2014). One large drop (3 mm) with the equivalent volume of 1000 drops of 200 pm was found to be as effective as smaller droplets if the droplet was captured by the axil of a cereal, for several systemic fungicides (Hislop, 1987). Larger droplets are less likely to evaporate during application and after contact with the plant. Extended drying time improves uptake and translocation of systemic and translaminar fungicides within the plant.

Droplets that are too big (>300 µm) can bounce, shatter or run off the target, reducing fungicide coverage. Not only that, but there are fewer droplets overall because small differences in droplet size (diameter) translate into large changes in droplet weight and volume. In fact, the equation to relate volume (V) to diameter (D) is:

$$V = \frac{1}{6}\pi D^3$$

And at equal volume, halving the droplet diameter creates eight times the droplets:

$$\frac{V_1}{V_2} = \frac{D_1^3}{0.5D_1^3} = \frac{1^3}{0.5^3} = \frac{1}{0.125} = 8$$

Droplet size is a measure of the surface area of the fluid being sprayed and directly relates to coverage. Reducing a droplet from 500 to 250 µm decreases both its weight and volume by 800% or eight times; reducing a droplet size by 50% doubles the surface area; reducing a droplet size by 25% creates a fourfold increase in coverage (Fig. 9.13). The smaller the droplet size the greater the surface area of the spray for any given volume of fluid.

Despite the advantages of larger droplets, most fungicide applications recommend medium to small spray droplets (Syngenta Crop Protection AG, 2002), which tend to adhere better and not run off, thereby providing better coverage and, by extension, better protection. This increase in coverage assumes the target captures the fungicide. Unfortunately, drift is only one of

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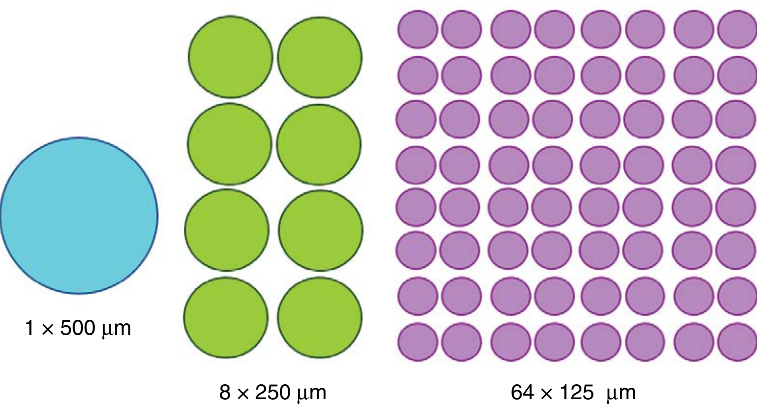

Fig. 9.13. The relationship between droplet size and volume demonstrates how mean droplet size is a measure of the surface area of the fluid being sprayed and directly relates to coverage. (Authors' own figure.)

many potentially bad outcomes that can result in fungicide failure. Of the remaining droplets out of a population of droplets, the fraction that captures the target has four potential outcomes on impact: (i) the droplet adheres to the surface; (ii) it bounces off the surface of the target; (iii) it shatters on impact; or (iv) it runs off. Three major components of this process interact to influence outcome, including the kinetic energy (mass and velocity) of the droplet, the surface tension of the liquid and the plant surface. Greater kinetic energy due to larger droplets, higher pressure or faster ground speed results in larger spread of the droplet on impact. This spread can be increased by reducing the surface tension of the liquid, increasing both the spread and the likelihood that the droplet is retained, and is the reason why surfactants, spreaders and wetting agents are often used in the formulation of fungicides. However, this same droplet can be dislodged during high wind, or if the surface becomes saturated with too many droplets (excess volume), and result in runoff. Adjuvants described as stickers work to reduce surface tension and the possibility of runoff, but they may impede coverage. For a successful fungicide application to plants, the droplets must be large enough to prevent drift and evaporation, but not so large as to run off, shatter or bounce off the target. To achieve this for foliar applications with sparse canopies, small to medium droplets are usually preferable, assuming they contact the target; for internal deposition in a dense canopy, larger droplets are needed for penetration; and to reach lower in the canopy or the soil surface (sprench), coarse to very coarse droplet sizes provide improved lower canopy and crown protection along with uptake by roots or tubers.

To assess droplet size and density, water-sensitive paper is used. Water-sensitive paper has a treated yellow surface that stains blue when contacted by aqueous droplets (Syngenta Crop Protection AG, 2002), negating the need for colorimetric dye. The use of water-sensitive paper attached to representative leaves throughout the plant canopy assists in monitoring the coverage and should be used both in calibration and in actual pesticide applications. These cards permit the visualization of spray droplets, patterns and density, similar to what were received by the leaf.

Adjustments should be made in subsequent applications to try to improve the results. Spray samples are compared with a known standard. The manufacturer of the cards suggests that 50 to 70 droplets/cm^2 for fungicide applications are necessary to provide satisfactory results, much higher than what was found for control of black sigatoka ascospores (*Mycosphaerella fijiensis*; MYCOFI) on banana leaves by mancozeb or chlorothalonil at 10 droplets/cm^2, VMD = 602 µm (Washington, 1997). Droplet density alone does not determine the efficacy of a deposit, but also the timing of the application, how available the deposit is for control and the thoroughness of coverage. Several studies have shown that contact fungicide efficacy increases with coverage on a diversity of crops (Grinstein *et al.*, 1997;

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Washington, 1997; Gent *et al.*, 2003; Abbott and Beckerman, 2018), that efficacy improves with decreasing droplet size (range 90–140 μm) and decreasing concentration (Cross and Berrie, 1995), and that systemics are less influenced than contact plant protection products (Prokop and Veverka, 2006) due to the translocation and movement of the systemic AI. For both systemic and contact fungicides, a zone of inhibition was found to surround the droplet deposit and could be recognized by the presence of ungerminated spores (Washington, 1997; Ypema and Gold, 1999; Bartlett *et al.*, 2002).

Spray pressure

Droplet size can be selected via spray pressure (higher pressure reduces droplet size) or nozzle choice. With PTO-driven sprayers, slower speeds result in lower pressures, spray pattern deterioration and poor coverage; higher speeds can create too fine a mist that drifts, also reducing efficacy. Speed maintenance is essential in PTO sprayers but is often difficult if the topography is uneven or the soil is waterlogged.

Increasing sprayer pressure often improves nozzle operation, resulting in improved coverage and deposition. However, it is important to remember that a complex interaction exists between droplet size, velocity and obstacles before the droplet reaches its target. A common misconception is that increasing pressure increases canopy penetration. Higher than recommended pressures will increase the delivery rate at the cost of reduced droplet size and spray pattern distortion, resulting in both spray drift and uneven coverage. Higher pressures increase droplet velocity, but this diminishes upon canopy entry. By the time the spray enters the canopy, the faster velocity is lost on impact, especially for the smaller droplets, resulting in a finer spray. Although smaller droplets can penetrate and reach the target, there is significant potential for crop damage from impact or abrasion, particularly for fruit and ornamental crops. On the other hand, reducing the spray pressure may result in an incomplete spray pattern.

Volume

An effective fungicide application must be of sufficient volume to deliver the droplets in the appropriate size, density and pesticide concentration to reach the target, and to provide thorough coverage of and adherence to the plant surfaces. Application volume is one variable that growers can use to improve efficacy and determines both the number of pesticide droplets produced and how well the target is covered. When the volume is too great or droplets are too large, shatter and runoff occur, resulting in an unprotected target; however, larger volumes reduce the risk of drift because coarser sprays with larger droplets are applied. Should the volume be insufficient, suboptimal or incomplete, the application will fail to protect the entire target population. In reality, the spray volume required to adequately cover different canopies will vary with the crop, crop age and density of the target canopy.

Due to the variation between canopy structure and sprayer mechanics, spray volume will always be a variable that the operator will need to consider at every fungicide application. In reality, the trade-off exists because sufficient spray volumes that ensure adequate deposition on inner canopy leaves and fruit will often result in runoff on the fruit and foliage in the outer canopy (Figs 9.14 and 9.15).

Volumes play an essential role in the pesticide application density, measured as drops per area. Curiously, the relationship between fungicide droplet and deposit density remains understudied and heavily relies on work done with insecticides (Washington, 1997). Studies that looked at deposit density of chlorothalonil and mancozeb for the control of MYCOFI found that a deposit density of 10 droplets/cm^2 with a VMD = 602 μm resulted in no germination of ascospores for both fungicides, whereas deposit densities of 2 droplets/cm^2 with a VMD = 989 μm and 5 droplets/cm^2 with a VMD = 804 μm resulted in significantly higher levels of germination (Washington, 1997). The author further cautioned that his results encompass only one aspect of fungicide efficacy and stated that ‘another important factor to consider when determining optimal droplet size for pesticide applications is penetration of the spray droplets into the plant canopy’ (Washington, 1997). Fortunately, most fungicide applications in the field result in a broad spectrum of droplet sizes, thereby providing both coverage and penetration.

Volume was also found to impact the interface of the area between the droplets and

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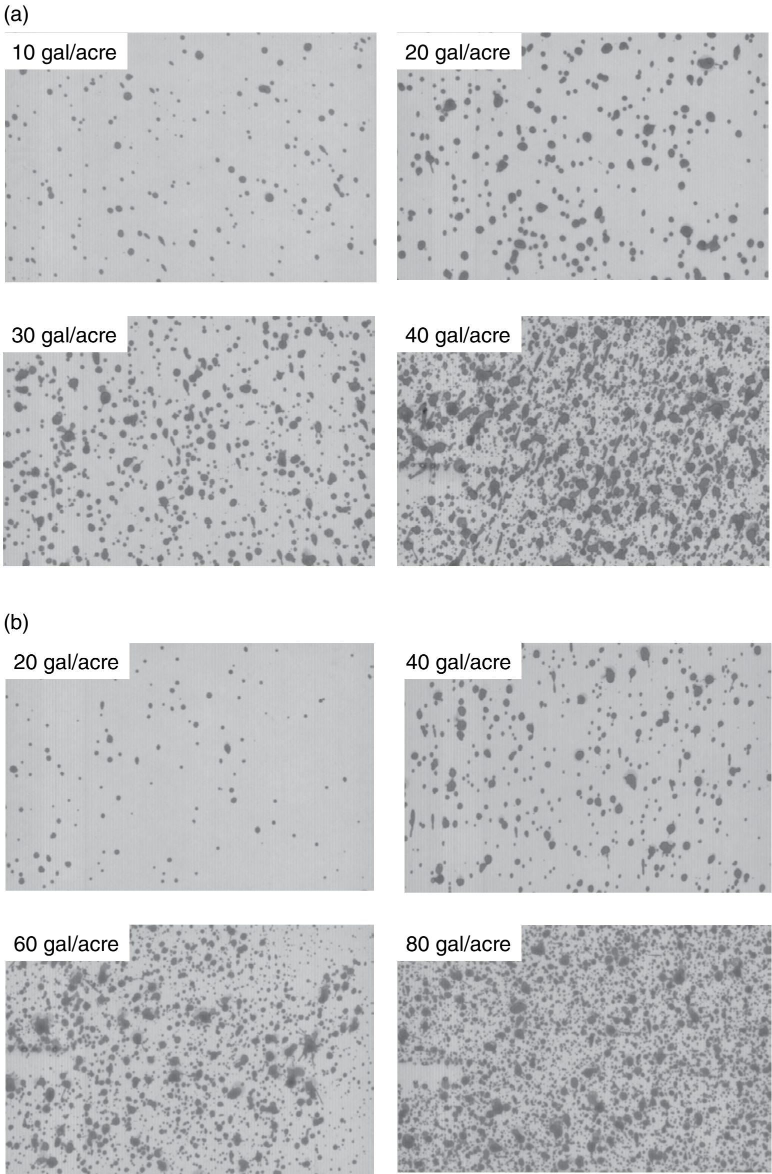

Fig. 9.14. Images of spray deposits on water-sensitive papers at 3 ft (0.9 m) height inside the canopies of (a) 2-year-old and (b) 3-year-old 'Autumn Spire' red maple liners at four different application rates; 1 gal/acre = 9.3540 l/ha. (From Zhu *et al*., 2011 with permission.)

the plant surface: as the volume increased, the droplet sizes were altered (Crabtree and Bukovac, 1980). Understanding the interaction between droplet size, concentration and volume is challenging, and involves another factor, droplet contact area, which is impacted by adjuvants, the target surface, volume and the environment, all of which influence spread.

In conclusion, droplet size, spray volume, droplet number and fungicide concentration all influence fungicide efficacy (Ebbert *et al*., 1999). When searching for answers on how to improve

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Fig. 9.15. Coverage difference resulting from two different nozzles. Coarse droplets (a) compared with the retention by medium droplets (b). Clear droplets are dew. Note the inconsistency in coverage and distribution. (From Shepard *et al*., 2006, used with permission.)

pesticide applications, many crop consultants often provide simple, succinct, straightforward statements on how to improve efficacy (e.g. increasing volume improves control, decreasing droplet size improves control, changing sprayer pressure, etc.). However, the diversity of plant hosts, fungal pathogens and environmental variables are all confounding factors that prohibit thoughtful people from ever proclaiming that changing a single variable will improve the outcome in every pathosystem.

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10

Fungicide Efficacy Evaluation

Key Points

- Fungicide efficacy evaluations include the direct efficacy of the fungicide against the target pathogen(s), the impact on the host, environmental impacts on efficacy, the economic benefits of the fungicide and the agronomic sustainability of the product.
- The primary goal of an efficacy trial is to objectively assess the effectiveness of the experimental fungicide against a specific pathogen that impacts a specific crop.
- The minimum effective dose is the smallest amount of product required for a sufficient level of efficacy against the target pathogen.
- It is easier to get a result about a product than it is to get an answer regarding efficacy.

Introduction

All pesticides, including fungicides, require evaluation with adequate and standardized procedures to evaluate efficacy and ensure that label claims, directions and use accurately reflect the results of the trials performed. Fungicide trials and their evaluations provide the necessary data to support the fungicide label recommendations. Trials performed for fungicide label development and registration include evaluating the test product against potential pathogens and crop combinations; using recommended equipment, methods of application, rates, timing and application numbers; and assessing potential incompatibilities with other products and the duration of disease management. The cost of these trials is primarily assumed by the company, and they are a large part of the registration process.

The components for evaluating fungicide efficacy include the *actual (direct) efficacy* of the fungicide against the *target pathogen/s*, the impact on the *host*, the role of the *environment* on efficacy, the *economic benefits* of the fungicide and the *agronomic sustainability* of the product. Fungicide efficacy evaluation is primarily focused on defining the *minimum effective dose*, the least amount of fungicide necessary and sufficient to control multiple targeted pathogens in a diversity of environmental and cropping situations. This is balanced against the potential negatives of crop phytotoxicity (Box 10.1), impact on pollinators, symbionts and natural enemies, and resistance management.

We define *actual efficacy* (or *direct efficacy*) as the degree of consistent control against a set level of disease for a prescribed period of time that is significantly better than the untreated and inoculated control. The overriding criterion is that the product must provide clear, significant and economically beneficial results to the grower. Inherent with the identification of efficacy is the determination of the minimum effective dose to protect or improve crop yield, quality, or

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Box 10.1. Phytotoxicity.

Phytotoxicity is defined as the temporary, long-term or permanent damage to plants due to the application of a pesticide (EPPO, 2014). Symptoms may impact a part of the plant or the entire plant and may include wilting, discoloration or death of plant tissue, deformity, retardation of emergence, development, flowering or fruit set, or even death of the entire plant. More severe symptoms are rarely observed in early efficacy trials; later trials or minor use studies may observe more subtle symptoms of phytotoxicity.

to provide a reduction in disease(s) or damage. Ideally, this dose becomes the minimum recommended dose on the product label and is identified as a balance between efficacy against the target pathogen while being safe for the applicator, consumer and environment. Based upon the diversity of pathogens along with varying environmental conditions where crops are grown, this minimum effective dose is often more a range than a specific number. The identification of this minimum effective dose or range of values is derived empirically based upon trials that evaluate a fungicide at a higher dose compared with a lower dose, the timing between applications and under varying conditions.

The data provided should be sufficient to permit an evaluation regarding the level, duration and consistency of disease control, the yield response and/or the effects on quality of the plant product. All caveats and the various conditions of use, such as the minimum effective dose (or range), treatment frequency, disease threshold levels (if available) and method of application, need to be stated. It is important to recognize that there is no one, prescribed level of control or benchmark to be attained, as different pathosystems are subjected to and sustain different levels of disease before an economic threshold is met. In some instances, very little disease is tolerated (e.g. ornamentals, fresh produce) whereas in other pathosystems with processed crops, a level of some disease is acceptable. Note that lower levels of disease control may be considered acceptable in some pathosystems, particularly if registration is for Organic or another specialty designation. All of this must be balanced against issues of phytotoxicity or loss of product quality. Product efficacy is evaluated against crop loss, quality reduction and unacceptable levels of *phytotoxicity* (Box 10.1). Ultimately, any evaluation of efficacy becomes an exercise in risk management and a trade-off between the positive results of the treatment (increase in quantity and/or quality) balanced against the risks of doing nothing (partial or total loss), while considering new risks of phytotoxicity, collateral damage to the environment and non-target organisms, and fungicide resistance.

Factors to be Considered When Evaluating Fungicides

The target pathogen

A foundation of plant pathology is the correct identification of the pathogen responsible for a given disease, including its Latin name to species and *forma specialis* if needed, along with the identification of any fungicide resistances. Without this information, trial data and successful disease management may be compromised.

A virulent pathogen is a necessary component of the disease triangle and fungicide efficacy trials, and a diversity of virulence better represents the real world where fungicides are used to protect crops. Trials require the use of different isolates, strains or races where these are likely to show different degrees of susceptibility to the product and virulence to the host plants. In this way, the work is very different from model systems which often review the results of one or two isolates on one or two clones or cultivars that provide a consistent response.

Efficacy evaluations need to be performed with disease that is at levels of economic importance. Treatments performed too early may not have been exposed to realistic disease pressure, whereas treatments provided too late may also fail to control epidemics or discriminate differences between treatments. In conducting trials, naturally occurring infection in trial blocks or plots is preferable, but not always feasible. In these instances where natural infection fails to reach sufficient levels, the addition of exogenous pathogen inoculum may be necessary. By the end of the trial, disease levels need to exceed a threshold to show clear evidence and acceptable efficacy against the pathogen. Quantification of

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inoculum and reproducibility of inoculation (culture media, conditions, quantitation of spores, etc.) should be consistent between the positive control (untreated, inoculated), negative control (untreated, inoculated with media alone) and inoculated treatment plots at the beginning prior to the first treatment.

The host

Effective fungicide trials require all components of the plant disease triangle to be present. Having a susceptible host is an obvious necessity; less clear is how susceptible the host must be to drive sufficient disease to evaluate efficacy. A host that is extremely susceptible may succumb so quickly to the pathogen that trials cannot discriminate between treatments. This is seen with specialty crops that have a history of reliance on clonal propagation. These extremely popular but pathogen-susceptible varieties have resulted in disease management challenges (e.g. certain varieties of wine grapes, apples, bananas, sweet cherries and potatoes). During product development, efficacy assessments, phytotoxicity and fungicide trials should be performed during key developmental (phenological) growth stages, particularly during sensitive or highly susceptible times such as flowering, fruit development or seedling/cutting establishment. Evaluation of the host should not be specific to one variety, as numerous trials have identified certain crop or cultivar sensitivities to some fungicides (e.g. certain apples to azoxystrobin; stone fruit to copper; soybean to prothioconazole and tebuconazole, among many others).

The environment

Just as highly susceptible or resistant hosts can skew trial data, the same is true for the environment. Maintaining consistent agronomic conditions within each trial site is the goal, but it is inevitable that soil and climate will vary within and between locations and can confound the results. This necessitates the use of multi-year, multiple-location trials to assess performance in a diversity of locales. Locations need to be documented precisely (e.g. coordinates). Latitude and longitude as measured with a Global Positioning System (GPS) is the preferred descriptor and is reported as degrees, minutes, seconds and decimal seconds with direction, or as degrees and decimal degrees with direction (e.g. latitude – 40°25′26.40″N or 40.4237°N, longitude – 86°55′44.40″W or 86.9212°W) (Schoeneberger *et al.*, 2012). Where trial conditions can be controlled (e.g. greenhouse, high tunnel or in storage) fewer trials are needed and sufficient data may be obtained more rapidly.

Locations should represent a diversity of environmental (soil, seasonality, climate) and crop conditions, according to conventional agronomic practices. Ideally, trials are conducted in locations where the product will be applied and where registration is desired. Obviously, trials cannot be performed in every location. When possible, trial results may be extrapolated and based on comparable (or more severe) conditions. When trial are conducted in a greenhouse, shade house, lathe (shade) house or storage facilities, conditions should be representative of those encountered under 'normal' use and practices. When trials must be conducted under specific environmental conditions, they are often performed in multiple locations and seasons.

During the trial, weather conditions should be measured and documented and include the temperature, precipitation (if outdoors) or irrigation, wind speed and direction, beginning with the treatment and/or inoculation and continuing until the trial is ended. Basic soil conditions, such as soil type, texture, pH, organic matter content, soil moisture and soil temperature, should be included. For greenhouse or storage trials, temperature and humidity should be recorded throughout the trial period.

Economic benefits

The use of a fungicide should provide obvious economic benefits to the grower that go beyond the biological efficacy and agronomic sustainability. However, highly effective fungicides may not be registered and labelled for specific uses for many reasons, including but not limited to an inability to recover the registration costs from the target area; the relevant crop may be grown on too small an area, or the cost–benefit analysis fails to deliver an increase in yield or quality to

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justify the cost of the fungicide. The relevant diseases may be sufficiently controlled by existing products. Fungicide companies are very wary of litigation if products are accused of failing to live up to expectation. Finally, countries and even states differ in the application of an economic or sustainability assessment for registration; these concepts may be directly assessed, implied or ignored, leaving the cost–benefit decision with the applicator.

Agronomic sustainability of product use

Fungicide resistance and assessment of risk represent one component of sustainability that is assessed in the labelling process. In the USA, crop profiles (e.g. Harrington and Good, 2000) are developed in collaboration with regional IPM centres and document the crop production and pest management practices on a regional or national basis for specific commodities. Other countries also take fungicide resistance into account in different ways.

As pathogen populations evolve, resistant isolates may increase as selection pressure favours those individuals that are less susceptible to a given fungicide compared with susceptible isolates. Over time efficacy is reduced or lost completely, as continued fungicide applications select resistant isolates. The evaluation of fungicide resistance risk may be part of the registration process to evaluate the risk of resistance and to identify strategies to prevent crop failure while managing, mitigating and minimizing fungicide resistance in a given pathogen population. Risk and uncertainty are inherent to agriculture, as is the risk of fungicide resistance whenever most fungicides are applied. This risk can be managed to a certain extent by the applicator and by their choices of what, when and how to apply the fungicide. Inappropriate use can result in increased applications but a loss of performance, resulting in economic injury and unnecessary, ineffective and potentially damaging pesticide loads in the environment. More on fungicide resistance can be found in Chapter 11, this volume.

Crop diversity is another aspect of *sustainability*. Multiple varieties of one crop, or many different types of similar crops, may be grown at the same time on neighbouring fields. In these situations, the impact of drift is part of the evaluation of a pesticide. This is usually only necessary if crops or varieties are particularly susceptible to phytotoxicity, or if the pesticide is to be applied in ways where there is a high risk of drift (e.g. via aircraft, application in greenhouse spaces, etc.). Examples of this include the risk of azoxystrobin drift on certain apple varieties (Fig. 10.1) and difenoconazole on some cowpea varieties. These data are often generated during direct efficacy trials. Fungicide trials to date have rarely included assessments of the possible impacts on natural enemies or pollinators, unlike insecticide trials where such data are collected. Thiophanate methyl, sulfur, lime sulfur and fluazinam are examples of AIs that are known to impact mite populations and fluopyram is documented to impact nematode populations. Impacts on non-target

Fig. 10.1. Azoxystrobin damage to young apple can be severe enough to cause fruit drop. (Photo credit: Win Cowgill with permission.)

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organisms, whether positive or negative, should be recorded whenever observed.

Efficacy trial components

Fungicide efficacy trials assess the effectiveness of fungicides against plant pathogens. The purpose of the trial is to assess if a product provides clear, significant and economically beneficial results to the grower (Box 10.2). In studies of efficacy, an *untreated inoculated control* (*positive control*) is perhaps the most important treatment and is included to demonstrate a significant level of disease that is expected to be consistently present throughout the trial. In this way, the positive control provides a reference point to determine the validity of the trial and what would happen in the absence of treatment. Its inclusion ensures that an adequate level of disease was present and that any reduction in incidence or severity is documented. If disease levels are low, efficacy cannot be clearly demonstrated (lack of distinction between treatments). Untreated, inoculated controls may be used to calculate efficacy levels, often in combination with an industry standard or reference product.

The positive control provides a contrast to identify the incidence and severity of infection, along with the reduction in quality and/or yield, in the absence of a fungicide. The positive control is contrasted against the *uninoculated, untreated control* (also called the *negative control*) which identifies the background level of disease that may have been present. In this way, the negative control is the uninoculated and untreated treatment, and provides a benchmark for plant quality and what a healthy plant is capable of yielding. It should not receive any of the treatments under study but be treated in the same measure as all other treatments (water, light, plant growth regulators, fertilizer, etc.). The negative control validates the positive control: in this case, how disease impacts plant quality and yield. The negative control provides assurance that plants were not infected prior to the start of the trial, provides information on pathogen spread and disease development, and identifies if additional exogenous inoculum is present in the study. These two controls serve as checks to assess the validity of the trial.

Often, but not always, product efficacy is compared against an industry standard fungicide or other disease management approaches (Box 10.3). Ideally, this would also include the use of other IPM tactics, including resistant varieties, GM varieties, cultural practices, the use of premixes (Box 10.4) and biological control. The standard fungicide provides a known degree of efficacy and serves as a further check to assess the quality of the trial and the fungicide applications. If new products show lower efficacy than the industry standard they will not be further developed. It is exceedingly important to choose the correct industry standard, i.e. one that is widely agreed to be the best current product to

Box 10.2. Can a less efficacious product be registered?

Not all products that undergo trials and development prove to be more efficacious. In fact, some fungicides may control a broader range of plant pathogens, even if the control is less intrinsically effective. Some products may compensate for a loss of efficacy by being more environmentally friendly, more amenable to cultural controls or IPM practices, or provide better protection upon reformulation. Lastly, with increasing issues of fungicide resistance in multiple cropping systems, many products that were once passed over for registration (e.g. polyoxin D, natamycin, some biologicals) have been developed due to a loss of efficacy by once standard products.

Box 10.3. Formulation changes.

When registering a product that has undergone a formulation change, but the AI remains the same, the number of trials may be reduced, and comparative trials can be used to demonstrate product equivalency.

Box 10.4. Premixes.

It is becoming increasingly common for industry to package multiple fungicides (or even multiple pesticides) together to reduce the need for tank-mixes. As with single minimum effective dose, premix fungicides require a justification for the ratio of AIs and the dose of these products. In many instances, synergy between compounds, or protection against rapid fungicide resistance, is promoted as the justification for this approach.

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control disease. An inappropriate standard (e.g. using mefenoxam to control an ascomycete) undermines the credibility of the trial performed. In some rare instances, an industry standard may not exist. In this case, efficacy is simply evaluated against the inoculated, untreated control.

Using validated reagents is important in all trials. Products should be freshly prepared and mixed with water of reasonable quality and close to neutral pH. Technical products may perform differently from formulated fungicides, and different formulations of the same AI may not perform the same way.

The criteria used to evaluate efficacy are determined for each country by the governing agencies. These agencies (e.g. Australian Pesticides and Veterinary Medicines Authority (AVPMA) for Australia; BPPO for Great Britain's Plant Protection Organization; Central Insecticide Board and Registration Committee (CIBRC) for India; EPA for the USA; European and Mediterranean Plant Protection Organization (EPPO) for the EU) decide what criteria are essential for fungicide registration and determine the acceptability of a given pesticide based upon each country's needs and the proposed use(s) of the products.

In order to establish efficacy in the field, the label must identify the dose that should be applied, the number of applications of a pesticide and the optimal timing of the pesticide. The identification of the minimum effective dose becomes a compromise between:

- the higher and lower dose;
- the persistence of the product;
- the number of applications;
- the number and diversity of targeted pathogens;
- crop systems; and
- the ability of the product to provide control.

All of these data are gathered via multiple trials (Table 10.1) focusing on the pathogens that cause the most damage to food security and economics, the mitigation of which provides the greatest agricultural and economic benefit. Within each trial, a series of different doses is utilized to demonstrate efficacy differences between the minimum effective dose, higher doses and lower doses. These may include dose series of 2×, 1×, 0.75× and 0.5× to evaluate. Additionally, trials include a diversity of environmental conditions, cultural practices, hosts (different cultivars or species) and pathogens, in addition to an industry standard.

Table 10.1. Basic number of direct efficacy trials required in an area of similar conditions that support the effectiveness of a product against a pathogen in a given pathosystem. (For further explanation, see EPPO, 2018.)

Use	Fully supportive results required
Major pest on major crop	10 (range 6–15)
Minor uses	3 (range 2–6)
Major pest; protected conditions	6 (range 4–8)

It is important to recognize that unlike drugs for human diseases, agricultural fungicides are employed for a wide diversity of fungal pathogens and hosts. It is impossible to provide evidence for the minimum effective dose for every situation. Data are simply required for the primary targets, in addition to other representative targets within similar cropping systems. Any observation of phytotoxicity may be included, as well.

Efficacy Trial Experimental Design

The physicist Richard Feynman probably described the challenge of a scientist best when he said: 'The first principle is that you must not fool yourself – and you are the easiest person to fool.' Proper experimental design is how you best prevent the confirmation of biases. Reproducible science and statistical tests are predicated upon the assumption that the design is correct. Statistical manipulation can provide statistical significance where none exists, but no actual scientific significance can be obtained in a poorly designed study. Trial objectives and evaluating criteria should be defined in advance of the experiment. Statistical analyses should be identified and assumptions that support their use should be declared as well. See the 'Further Reading' section below for some recommended statistical texts (Box *et al.*, 1978; Clewer and Scarisbrick, 2001; Field *et al.*, 2012; Frost, 2020).

The primary goal of an efficacy trial is to objectively assess the efficacy of the experimental fungicide against a specific pathogen that impacts

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a specific crop. This requires, at a minimum, the positive control, the product tested and an industry standard. The end goal of any fungicide efficacy evaluation is 'to ensure that the proposed claims and use recommendations on the product label are supported by trial data and reflect the actual performance of the product while providing a clear benefit to the user' (FAO, 2006). For fungicide efficacy studies, fungicides and other plant protection products are commonly evaluated by the reduction of crop loss or damage, protection and/or increase of crop yields or quality. This value is balanced against any negative effects of the fungicide, including phytotoxicity to the target, yield reductions, negative impacts on pollinators and beneficial organisms, the risk of fungicide resistance and any negative impacts on sustainability. It is worth reflecting that these are very different from the evaluations for 'plant health' and should not be conflated with plant protection.

To achieve the primary goal of an efficacy trial requires that the person evaluating the trial identifies and declares which variables are to be quantified. In most instances, fungicides are used to protect or increase crop yield compared with an untreated, inoculated or naturally infected control. Efficacy evaluations should identify any changes in yield, quality, processing or storage life. Other factors that may be evaluated include how a fungicide may affect the taste of both fresh and processed product. In some instances, harvested seeds need to be evaluated for their viability and vigour; cuttings need to be evaluated for the ability to root and subsequently grow; tubers, bulbs and rhizomes require assessments for the ability to sprout and grow.

Variables

The objective of a trial is to generate quantitative data which are then analysed to estimate the differences between treatments. Consulting with a statistician who has expertise in agricultural research prior to trial design, experimentation and data analysis is always recommended. The correct experimental design and statistical analysis allow the researcher to isolate and identify natural variation and to determine if this variation between treatments is real and not an artefact (Box 10.5). Experiments are designed to examine and evaluate a *variable*(s). A variable is something that is measured; it can also be controlled and manipulated.

There are two main types of variables: *categorical* and *continuous* (Table 10.2). These classes are often defined empirically during the process of measurement, and they are also defined by their mathematical properties (Stevens, 1946).

Box 10.5. Important statistics concepts.

A lot of understanding and misunderstanding regarding experimental design and hypothesis testing can be placed at the feet of three titans of statistics: Fisher, Neyman and Pearson (Perezgonzalez, 2015). Fisher (1925) defined the null hypothesis (H_0) – literally, a hypothesis of no effect; that is, no treatment effect on a response variable, no correlation between two variables, no interaction of two or more factors, and so on. Not only is this *not* the scientific hypothesis of interest, H_0 is usually the *opposite* of the scientific hypothesis. The goal of this test was to avoid type I (alpha) errors and rejecting the null hypothesis when it is true.

Hypothesis: biweekly myclobutanil applications on cucumbers reduce the powdery mildew severity compared with the control.
H_0, the null hypothesis: myclobutanil has no effect on powdery mildew.
H_a, the alternative hypothesis: myclobutanil reduces powdery mildew incidence.

Karl Popper was a philosopher of science who championed the idea that a hypothesis was not scientific unless it was falsifiable – that it could be tested in the real world by means of a prediction. Data are collected and analysed to determine the extent to which the data are consistent with H_0 (using a test statistic, which is a function of the data). If the test statistic is large, and P is less than the previously defined critical value, H_0 is not supported and we reject H_0 (Fisher, 1925; O'Brien and Castelloe, 2007).

Remember: how statisticians interpret the word 'significant' is very different from how everyone else does! In statistics, 'significance' simply means not due to chance (probably true and factual) and provides a summary about the data insofar as a specific null hypothesis – nothing more.

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Table 10.2. Summary table describing different types of data and how they can be analysed. (Authors' own table.)

	Categorical		Numerical	
Characteristic	Nominal	Ordinal	Interval	Ratio
Counts, frequency, chi-square	X	X	X	X
Values are ordered		X	X	X
Mode	X	X	X	X
Median		X	X	X
Mean			X	X
Quantification of difference between values			X	X
Values can be summed or subtracted			X	X
Values can be multiplied or divided				X
Has 'true zero' as a value				X

The level of measurement applied to variables determines what analyses you can conduct. Identical research questions with the same experimental design can require different statistical analyses depending upon how the dependent variable is categorized and/or measured. Statistical theory provides a multitude of guidelines regarding appropriate analysis; consultation with an applied statistician is always recommended before undertaking an experiment.

Categorical variables

A categorical variable (sometimes called a nominal variable) is one that has two or more categories, but there is no intrinsic ordering to the categories. In plant pathology, categorical variables are commonly assigned in fungicide efficacy trials. For categorical data, frequencies are analysed instead of quantitative responses.

Categorical variables can be further defined as nominal or ordinal:

- *Nominal* data labels a variable without providing a quantitative value (e.g. fungal species, plant species, fungicide, cultivar, viable/not viable). There is no hierarchy or ordering of nominal data, nor can it be measured. Typical descriptive statistics applied to nominal data are frequencies, percentages and chi-square. Dichotomous variables are nominal variables with two levels (e.g. yes/no, viable/not viable, effective/ineffective or Mat1-1/Mat1-2).
- *Ordinal* variables have two more categories that can be ordered or ranked. There is no numerical relationship between the orders (e.g. Likert scale, disease severity rating is 1 to 5; 1 = healthy, 2 = stunting/chlorosis, 3 = minor wilting, 4 = moderate/severe wilting, 5 = plant death). Some researchers treat variables measured with Likert scales (e.g. with labels such as 1 = strongly disagree, 2 = disagree, 3 = neutral, 4 = agree and 5 = strongly agree) as interval variables. However, treating Likert scale or disease severity responses as interval data carries the assumption that the differences between points on the scale are all equal, which is rarely true in fungicide trials.

Keep in mind that it is important to determine in advance the number of categories that a variable has.

Continuous variables

Continuous, numerical variables have a quantity that can be counted or measured and are therefore quantitative. A continuous variable can have an infinite number of possible values *except* when it is:

- a *ratio* – the variable has a meaningful zero-point (e.g. age, height, distance, 1.0 to –1.0); or
- an *interval* – measurement occurs via a continuum with fixed values between two points but does not have a meaningful zero-point (e.g. temperature measured in Celsius or Fahrenheit).

When developing a hypothesis, it is important to frame the hypothesis using terms (variables) that measure the concept. This allows the process

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to be further broken down into the questions: What variables will be used to test the question? What scales will be used to measure the variables?

A fungicide efficacy trial is an experiment (or a series of experiments) performed to evaluate the relationship between a fungicide treatment (also called *conditional variable*, *experimental variable* or *independent variable*), control (*baseline*) and an outcome variable (also called the *dependent variable*). In a basic fungicide trial, manipulating the treatment (conditional variable) is a tool used to understand how the treatment (fungicide) affects the outcome (e.g. yield, quality, appearance, mortality).

To objectively evaluate fungicide efficacy, trial data need to be analysed with the appropriate statistical tests to ensure that actual fungicide efficacy is distinguished from inherent experimental variability. For this to happen, the experiment needs to be designed to account for issues of sampling and randomization. This allows the scientist and the scientific community to draw valid conclusions and, in this case, improve disease management.

Plot layout

Plots are specific areas where treatments are applied and serve as the basic unit of the field research project (Box 10.6). Plots are often grouped into *blocks* (blocking) to improve the precision of comparisons between treatments. Blocks can be complete or incomplete: incomplete blocks simply do not have every treatment. Treatments are required to be randomized within each block to justify the usual assumptions for an analysis of variance (ANOVA). This helps ensure that treatment comparisons are unbiased.

> **Box 10.6.** Plot specifications.
>
> Experimental design and plot size are dependent upon the stage of the efficacy trial. Early-stage trials often use a small number of plants in a laboratory, growth chamber or greenhouse. Later, 'real world' trials must consider the crop and cropping system, conventional application techniques (e.g. backpack, boom or air blast sprayer; see 'Fungicide Application' section below and 'Sprayer Technology' section in Chapter 9, this volume), re-entry interval (REI), pre-harvest interval (PHI) and harvesting. In trials requiring the use of a boom or air blast sprayer, a buffer strip needs to be included, the width of the boom; for an air blast sprayer, multiple trees or vines are sprayed with only internal replicates counted to compensate for the inevitable drift that results; backpack sprayers may only need guard strips or other means to protect against drift.

Experimental designs can include, but are not limited to, randomized design, randomized block design, split plot and factorial design. The choice of the experimental design directly impacts trial power and the ability to detect both statistically significant and meaningful differences. The FAO (2006) stated that the number of residual *degrees of freedom*[1] in the experimental design should be at least 12. Degrees of freedom define the probability distribution of the test statistic (chi-square, F-distribution, t-distribution) and these distributions are used by hypothesis tests to calculate P values.

Rectangular plots often result from mechanically planted fields, facilitating mechanical harvesting (but also causing an increase in edge effects). Square plots minimize edge effects (compared with rectangular plots) but are often more of a challenge to lay out. Guard and buffer strips need to be accounted for when assessing and planning experiments. Ideally, plots should be similarly sized. A greater number of plants and larger plot sizes improve accuracy but are limited by the twin realities of economics and labour.

A completely *randomized design* is statistically the most powerful, meaning there is the maximum chance of detecting a significant difference if one exists. The purpose of randomization is to eliminate any potential biases that may skew treatments, whether done subconsciously or deliberately. Random does not mean chaotic or haphazard, but that every group receives equal likelihood of treatment. Since statistical procedures are based on the fundamental assumption that variation is random and determined by chance alone, this assumes that the trial environment is completely homogeneous. Should any heterogeneity exist within or between fields, differences may be detected that have nothing to do with product testing. For this reason, this design is rarely used in the field, and is often incorrectly used in greenhouse or laboratory experiments. Light, temperature and humidity gradients often exist in growth chambers, greenhouses and

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wherever experiments are conducted, even if it is a matter of a few degrees, lumens or per cent humidity. It is always good policy to randomize replicates and not put all the replicates of one treatment together.

To block or not to block?

A block is simply a group of plots within a homogeneous area; a block layout is designed to control any heterogeneity that exists between experimenters and/or within timing, the environment or treatment conditions, so that variability among plots within blocks is less than the variability between blocks. The completely randomized design works in uniform controlled conditions. In reality, these conditions are more difficult to obtain than is recognized. If there is variability across the site or growth chamber, a randomized block design is often the most appropriate for efficacy testing. Blocking is useful (and even essential) if other factors (light, moisture, soil conditions, temperature, etc.) introduce variation that might mask treatment effects. Where or when significant heterogeneity exists, a randomized block design takes this heterogeneity into account. Each treatment appears once per block; blocks are designed so that variation within blocks is less than variation among blocks. Plots within the blocks, or blocks themselves, may be adjacent to each other, but it is not a requirement.

Randomized complete block design

With a randomized complete block (RCB) design, each treatment occurs only one time in each block and the order of treatments (fungicides A (control), B, C and D) within the blocks is determined randomly (Table 10.3; most random designs are more random than the example provided). A strength of this design is that some treatments may be replicated more times than others.

Table 10.3. Fungicide treatments within a block. Note: this layout is actually a *Latin square*, in that each treatment appears only one time in each row and each column. (Authors' own table.)

Row 1	A	B	C	D
Row 2	D	C	A	B
Row 3	B	A	D	C
Row 4	C	D	B	A

Factorial design and split plots

Experiments with *factorial design* are used to evaluate the effect of two or more independent variables on a single dependent variable. This allows the experimenter to identify all possible interactions among and between variables (e.g. the total number of treatments (factors, or variables that affect the response of interest), how many levels each treatment has, and the number of experimental units in the design). For example, a 2^3 factorial experiment evaluates three main effects (factors) (A, B, C), three two-factor interaction effects (AB, BC, CA) and one three-factor interaction effect (ABC) for eight experimental units in the design; $2^3=8$ (a, b, ab, c, ac, bc, abc and 1). Keep in mind that factorial describes the statistical design. The experiment can still be plotted as a randomized or an RCB design.

Some factors cannot be easily manipulated or changed. The *split-plot design* is used when one factor (treatment) is intractable and/or when one treatment (factors) needs more replication (Table 10.4). It is commonly used to evaluate how different treatments (factors) interact. It is more efficient statistically speaking, but care must be taken to analyse data appropriately, and statistical analyses become more challenging with split plot due to the interaction between plot and treatments. Some programs do not have the capability to correctly analyse the data. The split-split plot design involves three or more treatments (factors). Each treatment has levels (which may or may not be discrete values). All combinations of these levels across all such factors are applied to the plots. In some cases, a multifactorial design is needed: for example, multiple doses of the same product are tested; identical doses on different cultivars; different combinations of tank-mixes, etc. A split-plot design is then often used, where the main plots

Table 10.4. A split-plot design to compare treatments in Organic and conventionally grown crops. (Authors' own table.)

	Organic		Conventional	
Block 1	D	C	A	B
Block 2	B	A	D	C
Block 3	C	D	B	A
Block 4	A	B	C	D

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are subdivided into subplots. The size of the subplots should be sufficient, however, to allow reliable treatment and evaluation.

Repeated measures

Many fungicide trials are performed under field conditions over a period of time. In these studies, the same plants are being measured more than once on the same dependent variable and are called repeated measures. For these trials, environmental conditions impact efficacy. In this case, performing these trials in multiple locations or at different times (replication in space and time) requires that efficacy assessments be blocked by space or time. Because the plants experience the experimental conditions they also serve as experimental blocks or as their own control in repeated-measures analysis, as successive disease assessments are strongly correlated with each other.

Individual plants serving as their own, repeated-measure block controls for the very factors that cause variability between subjects and, in this way, reduces the variance to that within subjects and *not* between subjects. Unlike *longitudinal data* where the dependent variable is measured at several points in time for each subject, often over a period of time, repeated measures can be performed over space.

Unfortunately, many agricultural studies have more complex data, including but not limited to *clustered data*, when the dependent variable is measured once for each subject, but the subjects can be further classified (e.g. by field, cultivar, fertilization level, etc.). Each *cluster* contains multiple observations, creating a 'nested' or 'hierarchical' structure within the cluster. Not surprisingly, observations within a cluster are more consistent (e.g. less variation) than observations between different clusters. Consider: values repeatedly measured in the same plant are expected to be more similar to each other than values from different plants and are not independent.

Power analysis

Statistical tests use observations to accept or reject the null hypothesis. *Power analysis* predicts the probability of detecting a statistically significant difference, should such a difference exist (avoiding a type I error, or alpha), or that the null hypothesis will be rejected when it should be accepted (thereby avoiding a type II error, or beta) (Box 10.7). In other words, the *power* of a study is the ability to identify an actual effect through a statistical test and distinguish it from 'chance'. It is possible that a small sample size may be sufficient to detect large effects. Small effects, however, require a large(r) sample size. With increasing sample size is an increase in the power of the test: larger sample numbers translate into more data, increasing power and further protecting from type I and type II errors. If the difference between treatments is small, the trial will require higher power to distinguish between treatments. It is generally accepted that power should be 0.8 or greater; that is, you should have an 80% or greater chance of finding a statistically significant difference when there is one. If no statistically significant differences are observed between a positive control, industry standard/reference product and the test product/treatment, the trial may not have been sufficiently powered to distinguish the difference and is inconclusive. It is important to stress that statistically inconclusive is not synonymous with ineffective or bad, any more than statistically significant means that something is good or effective, and that non-significant findings are not necessarily the same thing as no difference (Altman and Bland, 1995). Remember: power is simply the probability that a hypothesis test will detect an effect in a sample derived from a broader population. As such, it is always possible that the effect of interest may not exist in the sample that was drawn from that broader population. Larger sample

Box 10.7. Type I and type II errors

Most of us were told the fable about the boy who cried wolf. This boy caused both a type I and a type II error. When he called 'Wolf' the first time, everyone believed there was a wolf even though there wasn't. In other words, a false positive or a type I error. Later when there actually was a wolf, the boy yelled 'Wolf' but no one believed him, committing a type II error or a false negative, thinking there wasn't a wolf when in fact there was. When confused, substitute 'wolf' for the effect that is being hypothesized.

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sizes are no guarantee of good data if the experiment is poorly designed, and it is possible to draw confident conclusions that are well powered but inaccurate. Finally, power analysis can be applied a priori, before an experiment, to identify the likelihood of identifying significance, or it can be used after the fact (*ex post facto*) to determine if the results were realistic and not spurious.

To detect any given meaningful difference, the power of a trial is higher if residual variation in the results (i.e. the variation not caused by the treatments) is smaller or if the number of replicates is larger. As a rule of the thumb, a trial should include a minimum of four replicates per treatment (FAO, 2006): too few replicates result in insufficient information, imprecise estimates and low statistical power. Unfortunately, many research questions are underpowered, meaning not enough replications were included and the experiment was not repeated enough times (Gent *et al.*, 2018). In fact, recent soul searching in the sciences has brought the issue of reproducibility front and centre (National Academies of Sciences, Engineering, and Medicine, 2019). The power and effect size of a study represent the likelihood that it will identify and distinguish statistical significance from background (noise) and if an effect of a certain size is due to chance. The probability of replicating this study is a function of sample size and effect size. Unfortunately, the exact number of replicates needs to be balanced between the power needed to discern differences, the variability in the target pathogen populations, and the dual realities of labour and economics. Remember, though, that with any statistical test exists the possibility that a difference is detected between groups that does not really exist.

Three factors influence the magnitude of the power: the number of observations (replication), the effect size in the population and the level of significance. Replication identifies an estimate of the experimental variability (unfortunately, this is often called by the pejorative 'error' instead of simply 'deviation' or 'variability') that can be determined for an experiment and is used to calculate degrees of freedom. Degrees of freedom define the relationship between the data relative to the number of properties to be estimated. In this way, more replicates increase the degrees of freedom and give a more precise estimate of effect size, how accurate the estimates are and provide quality controls describing how the experiments were conducted.

Effect size[2] is the quantitative measurement of the relationship between two (or more) variables in a population studied, along with the context surrounding the magnitude of any observed differences. In other words, effect size informs whether the relationship is strong or weak. In general, larger sample sizes increase statistical power (the 'strength' of a test) and improve the ability to detect effect size. It is effect size that distinguishes the magnitude of difference between treatments, regardless of sample size (Gent *et al.*, 2018). Cohen (1988) developed effect size index tables for several common statistical tests and provided definitions of what constitutes small through large effects (Table 10.5). Taken together, effect size identifies the magnitude of the observed effect or relationship between variables; the significance test identifies the likelihood that the effect or relationship is due to chance.

For fungicide trials, effect size informs whether a variable has a strong or weak impact on the outcome. Whereas a *P* value can tell you if the difference is statistically significant, the *r* value of the effect size tells you if the improvement is substantial or inconsequential relative to

Table 10.5. Cohen's *f* test. Scientists are usually satisfied when the statistical power is 0.8 or higher, corresponding to an 80% chance that an effect is real (Cohen, 1988).

	Effect size			
	Denoted	Small	Medium	Large
t-Test for means	d	0.2	0.5	0.8
t-Test for correlation	r	0.1	0.3	0.5
F-test for regression	f^2	0.02	0.15	0.35
F-test for ANOVA	f	0.1	0.25	0.4
Chi-square test	w	0.1	0.3	0.5

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the standard treatment and/or control. When performing trials, be sure to examine effect sizes and confidence intervals: 'These convey what a *P* value does not: the magnitude and relative importance of an effect' (Nuzzo, 2014). This isn't to denigrate the role of *P* value, but to put it back in its proper place as a partner (instead of penultimate determinant) of a process that involves additional data and scientific expertise.

Samples and sampling

Statistics are used to study samples which are a subset and/or a percentage of the entire population. The *sample* (also called a *representative sample*) is selected to represent all units in a population of interest. The sample size is denoted by *n*. In general, sample size increases the precision of estimates of various properties of the population. Since studying an entire population is not feasible due to issues of time and cost, a sample provides a snapshot from a part of the population that represents how the population is expected to perform. Regardless of your question, a representational sample size is a foundation of good experimental design. *What you sample, how you sample, when you sample and how you identify your sample size are all important*. What is the goal of your project? In order to achieve this goal, how many samples must be tested to reliably draw conclusions about the phenomenon being investigated? This is the measurable objective you take to address your question.

Several components factor into what constitutes an effective sample size, chief among which is the degree of variability being measured. Simple random sampling assumes that each element in the population has an equal probability of being selected to the sample (independent measurements). As such, the sample does not have to be that large to adequately represent the population. Data obtained from the sample can be applied to the development of inferences about the whole population. To do this correctly, a sample size must be *sufficiently* large. *In other words, you need to calculate an effective or adequate sample size*. More often than not, limitations in time and money result in a smaller than ideal sample size, underpowering the study. Be sure to clearly acknowledge this and any other limitation(s) when interpreting the results and drawing conclusions.

Often times, to drive the diffusion of innovation, trials are performed on-farm and in collaborations with farmers to demonstrate efficacy and quickly disseminate results to growers in their own fields. These demonstration plots are often designed as split plots and are comparisons of the experimental treatment versus current growing practice. These trials are often underpowered and not necessarily suitable for efficacy evaluation, but provide growers with personal observation of efficacy, a valuable component that facilitates the adoption of new practices.

Remember that statistics merely functions as a mathematical tool to identify if your observations are consistent, which is not necessarily the same as true or meaningful. Real-world significance can be very distinct from statistical significance (Altman and Bland, 1995).

Fungicide application

As the trial process progresses, the products are applied under conventional agricultural practices to establish a level of reliable efficacy. At this stage, the dose of the fungicide, the dilution rate and the volume delivered represent what will be on the label, unless this is earlier in the process and dose is still being evaluated. Application timing and frequency of application also need to be determined to identify acceptable intervals between applications that do not impact control.

Ideally, application should resemble conditions in the field, and adhere to the label. This certainly occurs later in the trial process; earlier in development, small-scale trials may be performed with hand-held equipment. In all instances, application equipment must be calibrated (see 'Sprayer Technology' section in Chapter 9, this volume) to deliver the appropriate rate. This requires calibration of volume, sprayer speed and, by extension, sprayer pressure, nozzle and droplet size to deliver the appropriate dose and coverage.

Efficacy assessments

There are a number of protocols and strategies for estimating efficacy. These range from informal methods of growers using a product for the first time, to large-scale, multi-state randomized

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research trials with complex methodological designs. There is no one protocol that is universally applicable for every fungicide trial; simpler or less complex methods may be more appropriate than the more complex or sophisticated approach. More often than not, combinations of techniques are used depending upon:

- the variables chosen (incidence and severity of the pathogen, infection levels, percentage mortality or control, yield, quality, etc.);
- the crop; and
- the pest being studied.

For any trial, baselines (pre-treatment assessments) need to be identified to provide for corrections to variable assessments (e.g. population reduction, corrected mortality, corrected incidence, etc.). With few exceptions, trials have been performed in every pathosystem; previously published studies on sampling and assessment methods can and should be consulted when possible. Regardless of the assessment method chosen, the sampling, rating methodology and statistical analyses should be described clearly and in sufficient detail to permit replication by subsequent researchers based upon the information provided in the report.

Statistical analysis

The data from a fungicide trial, regardless of where it is performed, should be quantified[3] and statistically analysed. Only then can decisions and judgements be made regarding the utility of the data. Unlike mathematics, statistics deals with uncertainty, such as measurement error, missing data, confounding variables, etc. Statistics recognizes that information is a work in progress and not yet complete; the solution depends upon how this partial information is inferred. Confidence intervals, probability, P values, t-tests, etc. are all used to account for the uncertainty. In this way, science operates like statistics was intended to, as a process of reducing uncertainty – and get the answer less wrong over time.

In a number of scientific publications, trials are designed to provide proof of concept – in other words, minimize uncertainty. In many instances, aspects of the trial may be unrealistic: in the amount of a product used to provide protection, the number of applications or the strict conditions required to get a result. In all these types of experiments, and in fungicide trials in particular, it is important that the trial be conducted to generate useful data. This means, as was previously mentioned, that the trial needs to have a level of disease that is sufficient to generate statistical differences between the treatments, along with the positive control. Included within the treatments are a standard that provides a reference for the level of control currently obtained and a negative control (uninoculated, untreated) to know what 'normal' is.

Plotting the data

Descriptive statistics can be used to summarize your data and provide a graphical overview of what is occurring. Properties like central tendency, distribution, measures of variability and outlier identification provide a snapshot that describes the data. Charts of these data are often more intuitive. The best practice is to use graphs and statistical output together to maximize your understanding of your data.

Central tendencies (mean, median, mode)

The mean, median and mode are all measures that describe where most of the values in the data set occur (central tendency). This single value is used to provide a snapshot that describes a data set by indicating the central position within that set of data (also called central location and summary statistics).

- *Mean*: the most commonly used, this is the sum of all observations divided by the number of observations. It can be used with both discrete and continuous data and serves as a model of the data set (even though it may not appear as an actual value within the data set). The use of means is most appropriate when the data have a symmetrical distribution and is very sensitive to the impact of outliers.
- *Median*: this value splits the data in half after it has been arranged in order of value or magnitude, with half of the values greater than the median and the other half of the values less than the median. The median is

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less susceptible to influence by outliers and skewed data.

- *Mode*: this is the value that occurs most often in the data and is used most often for categorical and ordinal data.

Measures of dispersion (standard deviation, variance, range)

Mean, median and mode are models of central tendency. Measures of dispersion are used to describe the variation within this sample or population and provide a check to evaluate how well the mean actually represents the data. Standard deviation, variance and range are measures of data dispersion that indicate the spacing or clustering of the data points fall around that centre. As these values increase, the data points spread out and disperse.

- *Standard deviation* is the square root of the variance and uses the original units of the data, simplifying data interpretation. Closely grouped data points have a smaller standard deviation than dispersed data points where the standard distance from the mean is greater.
- *Variance* describes how diverse a sample is by calculating the average squared difference of the values from the mean.
- *Range* is a measure of spread that describes the difference between the highest and lowest values in a data set. As it is based upon only two, and the most extreme, values of a data set, it is very susceptible to outliers. Furthermore, as sample size increases, range often expands. As a result, when comparing ranges, it is important to compare similar, if not identical sample sizes.

Lastly, the *area under the disease progress curve* (AUDPC) is a simple, but powerful single quantitative variable that describes disease intensity over time, and permits comparison among treatments, across locations and over years (Sparks *et al.*, 2008). It was first used in 1974 (Wilcoxson *et al.*, 1975) and is calculated *without regard to curve shape* (Shaner and Finney, 1977). This last caveat is important as any crop loss that occurs at a specific point in the outbreak (e.g. during the bloom, boot, fill, harvest, etc.) often results in an AUDPC that does not adequately reflect the outbreak or management strategies.

Analysis of variance

ANOVA is simply a special type of regression analysis that compares the ratio of systematic variance to unsystematic variance. Most fungicide trials consist of evaluating three or more different treatments, making a *t*-test unacceptable for data analysis. After obtaining the means for each treatment, to determine if these means are different ANOVA will be performed, followed by an *F*-test or *F*-statistic, the ratio between the two variances, to determine if any of the differences between the means are statistically significant. In one-way ANOVA:

$$F = \frac{\text{between-group variance}}{\text{within-group variance}}$$

ANOVA assumes that the variance of individual errors is the same across treatments, that individual error terms are normally distributed or normally distributed within each group, and that they are independent. Treatments should have a similar number of replicates (although modifications can be performed using Welch's *F*-test). One-way ANOVA evaluates one factor whereas two-way ANOVA, as the name suggests, includes a second factor.

If the *P* value from the ANOVA *F*-test is less than the level of significance, the null hypothesis is rejected, because not all group means are equal. ANOVA does not identify which groups may be different from other groups. For that, post hoc tests are required.

Replication and reproducibility

'Reproducibility and replicability are often cited as hallmarks of good science' (National Academies of Sciences, Engineering, and Medicine, 2019). The terms 'replication' and 'reproducibility' are often (incorrectly) used to mean a number of different, similar and even conflicting things (for a deep dive of this discussion, see Barba, 2018). The lack of clarity in usage predicates the lack of clarity of the issue in science. For our purposes, *replicability* means obtaining consistent results across studies aimed at answering the same scientific question, each of which has obtained its own data under identical conditions. *Reproducibility* means obtaining consistent results using the same processes (cultivars, treatments, materials, methods, conditions of analysis, code, input data, etc.). Experimental conditions may vary

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slightly, but the phenomenon is predicted to recur. With any study, the *probability of replicating a result exists as a function of sample size and effect size*.

To demonstrate the efficacy of a given fungicide, trials are performed in different locations with unique climates; they are often performed over the course of years and at different times of year. For these types of trial series (also called multi-site or multi-year trials), trial reproducibility is paramount to determine product crop safety, efficacy, minimum effective dose, product use profile, etc. Although reproducibility is essential, the number of trials necessary to establish this is not predetermined and varies depending upon: (i) the incidence and/or severity of the pathogen being targeted and the levels of disease being sufficiently high to discriminate differences; (ii) the importance of the crop in a given locale; and (iii) if phytotoxicity has presented a problem, on certain cultivars of a given crop. Simply stated, more trials are necessary for human staples (maize, wheat, soybean, etc.) and fewer trials are necessary for minor uses (see 'Minor use' section below). Multi-year trials that provide relative consistency of results may require fewer studies compared with a highly variable trial portfolio. At a minimum, expect approximately ten trials over two years, with additional trials performed as needed due to issues of experimental variability. Ultimately, the number of trials required is determined by the registration agency of each country.

Of course, there are caveats: some products are considered second-, third- or fourth-generation chemistries – variations on products that have accumulated extensive prior knowledge of the product's use may allow for extrapolation to other closely related products. Products that were brought to market in another country may have extensive data associated with that country and may require fewer trials to be registered in additional countries (or more, depending upon the agency, the country and the population's perception of pesticides).

Efficacy Evaluation for Minor Use Applications

For fungicides, 'minor use' is a term applied when the crop is of limited economic importance at national level, or the disease outbreak is episodic and limited in geography. These trials and crops were given the unfortunate designation of 'minor uses'. What is considered minor use in one country, state or province may be considerable in another one. The grower contending with the plant disease certainly does not see the problem as minor. Many specialty crops, including fruit, vegetables, herbs, ornamentals, or any crop grown on limited acreage or in a non-industrialized fashion, may also require the minor use of a plant protection product, even though the crop may be highly valued.

The designation of 'minor use' rests with the registration authority. Minor use encompasses those plant protection products that, although necessary for the growers of these crops, the projected sales would not make it economically feasible for companies to absorb the cost of full registration. In the USA and Canada, this gap was addressed with the IR4 project to provide a venue to develop and expand products for the growers of specialty crops; in Europe EPPO and in Australia APVMA oversee similar processes. First and foremost, the extension of an existing registration to a 'minor use' one requires the absence of phytotoxicity on the 'minor crop', while demonstrating effectiveness of the product against the target pest. When the target pest of the crop is of minor importance, fewer trials may be required. This is particularly the case once the direct efficacy has been demonstrated against a relevant major pest or against the same pest on a major crop, and extrapolation to the minor use situation is possible, such as from a food crop to an ornamental one (e.g. apple or pear to crab apple or flowering pear; sweet potato to sweet potato vine, etc.). However, additional testing regarding PHI may be required, particularly when the registration of a product is changed from a field crop to a fruit or vegetable crop (e.g. soybean grown as a field crop versus soybean grown as a vegetable crop (edamame); field maize to popcorn or sweetcorn).

Reproducibility, Record Keeping and Reporting

The reproducibility of a study is directly tied to the record keeping of the study. Records of a

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given study should be as complete, accurate and coherent as possible, and with enough detail to allow another scientist to replicate or reproduce the study and obtain the same or similar results. Successful record keeping protects research integrity and accountability. These records are held by the company holding the registration; as many of these studies are performed by young or newer scientists, fungicide trials serve as an excellent teaching tool to instruct these scientists on proper experimental design, statistical analyses, and reporting and record keeping. Records can be written, electronic or some combination thereof. All records should legible, well-organized, clear, concise and complete. Records should be secure and backed up, particularly electronic records.

Conclusion

The reality is that it is much easier to get a result about how a product performs than it is to get an answer regarding product efficacy, particularly when multiple crops and pathosystems are involved. Even with the most controlled and tightly reproduced trials, it is important to recognize and accept that the data are often variable, and that the results are incomplete and uncertain. Every result in the trial process (and science) is a temporary truth, subject to change upon new and additional information, different management practices and with pathogen evolution over time. This is a strength of science and not a weakness: our knowledge improves with the addition of more information. However, with this improvement comes the loss of a simple answer.

Fortunately or unfortunately, information is not a scarce commodity. In fact, there is too much of it, and most is not useful for making any decision, let alone a disease management one. Fungicide trials provide specific information about product performance, in one location at a specific point in time. The purpose of this chapter was to assist in making the information useful and aid in the creation of that knowledge. To do this, we need to recognize that we all perceive data subjectively, we parse these data selectively in keeping with our biases, and we have little recognition or self-awareness regarding the distortions that we are (knowingly and unknowingly) complicit in. Worse still, even if we have the knowledge needed to address plant disease management issues, we are often forced to speak in disclaimers, use jargon and/or hide behind technicalities to remain 'technically' correct – burying whatever kernel of knowledge we once possessed under a mountain of caveats.

Despite all these issues, knowledge is generated through the careful questioning of the data and by the scientists of themselves. There are four questions a scientist might want to ask after a fungicide trial: 'What is the evidence regarding this product?', 'What are my biases impacting this evidence?' (Box 10.8), 'What should I believe about this evidence?' and 'What should I do?' No one expert, statistical test or method can answer all these questions, which leaves us with a final question: 'How can we apply our judgement to the data – without capitulating to our own biases?'

The first step is to quantify the data and provide the context. To do this, we also must recognize that our ability to analyse data has grown beyond our ability to understand the results. Plugging numbers into a program or script is not going to generate knowledge without context, even with the appropriate analyses. The numbers, *P* values or *F* values or whatever, are not omniscient, and the only meaning these numbers have comes from whatever value we assign them. It is important that we realize this and check our bias every step of the way. The scientific discussion begins with the numbers – but it doesn't end with them. To improve the value of these numbers, we need to understand and evaluate any biases or logical fallacies that may have entered into the analysis. The key to developing this knowledge and improving plant disease management practices is being self-aware of our understanding of the system we are analysing in conjunction with recognizing our own biases. Only then can we provide value to our information and develop genuine knowledge to share with others. 'The improvement of understanding is for two ends: first our own increase of knowledge; secondly to enable us to deliver that knowledge to others' (John Locke).

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Box 10.8. Cognitive biases and logical fallacies in science.

It takes a heightened sense of intellectual humility to not fall prey to all the cognitive biases and logical fallacies that humans succumb to. It is important to recognize that at any point in time, we are susceptible to these 'brain bugs' and that they influence our thinking consciously and subconsciously (Kahneman, 2013; Damer, 2012). Every day, information is available – how we interpret and scaffold this information is influenced by how it is delivered, framed, received and incorporated into personal knowledge.

To be objective requires a conscious process to control for those influences that manipulate information, both externally and internally (especially internally!). The processes that can mislead the unwary have been given many names such as:

Anecdata: using personal experience to override larger data sets and dismiss statistics.
Appealing to nature or popularity as a form of validation: as plant pathologists we should be aware that nature produces some very toxic substances; perhaps none is as toxic as popularity.
Availability heuristic: judgements are influenced by what first jumps to mind (Tversky and Kahneman, 1973). First impressions and judgements are only first, and not necessarily the best or even accurate.
Belief bias: using a conclusion to support pre-existing beliefs. Without questioning these beliefs, they become self-reinforcing and even more impervious to challenge.
Cherry-picking: selecting the data to support an argument, while not recognizing conscious or subconscious efforts to suppress data that conflicts with a bias.
Confirmation bias: cherry picking (above) and/or interpreting new evidence as support for a previous belief.
False causality: presuming that a relationship between things is causal, as opposed to correlated or even spurious.
Framing effect: impacts decision making by how information is presented instead of by the information alone.
Halo effect: judgements are often influenced by previous interactions. Positive interactions spill over to associated interactions. In plant pathology, the halo effect can impact how a scientist evaluates a product based upon its manufacturer, the AI, the formulation, its application, the reports of other scientists, etc.
Straw man: in rhetoric, misrepresenting an argument to make it easier to attack. In fungicide trials, it could be using a false standard (e.g. using a triazole to control an oomycete, using a product for downy mildew to control powdery mildew, etc.) to support a different, but not necessarily more effective, product.

Notes

[1] Degrees of freedom is a number that estimates how many values can be varied in a data set. In other words, the degrees of freedom are the number independent pieces of information that went into calculating the estimate.
[2] The effect is the mean difference between outcomes comparing a treatment group with the control group. A *t*-test (two groups) or ANOVA (three or more groups) is applied to: (i) ascertain if an effect exists; and (ii) estimate the effect size.
[3] The type of variable recorded determines which statistical analysis can be correctly applied to analyse the data. Quantitative data are preferable in that more robust, parametric statistical methods can be applied, predicated on ANOVA. In the event that qualitative variables are used, less robust (but still informative) non-parametric methods are appropriate.

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11

Fungicide Resistance

Key Points

- Fungicide resistance is a critical factor in the development and use of fungicides.
- Resistance affects most current fungicide classes.
- The evolution of resistance is a classic case of natural selection.
- Three mechanisms account for resistance: target site modification, target site overexpression and efflux pump overexpression.
- Fungicide detoxification and metabolization is suspected as a fourth mechanism.
- Fungicide resistance can be managed by careful use of integrated disease management (IDM) principles, by using the minimum effective doses and employing mixtures and alternations of fungicides.

Introduction

Resistance to fungicides has grown in importance over the last 40 years and now ranks as one of the central preoccupations of the fungicide industry. Resistance emerged as a practical problem as recently as 1970 and has built steadily in the decades since. The incidence of resistance is essentially restricted to systemic fungicides that operate against single biochemical targets (single-site inhibitors). These were introduced from the mid-1960s onwards and include most of the major newer groups of fungicides (Table 11.1).

Resistance to fungicides is manifested as failures of previously efficacious products to control disease. It may be that the fungicide suddenly fails to provide any useful control at all. More commonly there is a gradual loss of efficacy such that larger doses and more frequent applications are needed to achieve adequate disease control. This can be due to either a resistant population replacing a sensitive one over the course of several growing seasons or from the stepwise loss of efficacy as isolates evolve multiple mutations. Whatever the mechanism, resistance removes the entire economic rationale of fungicide use as farmers derive no useful disease control. The cost of the product and time and effort spent in spraying the chemical are wasted. The economically valuable life of a fungicide, which is already limited because of the short period of patent protection, may well be further curtailed.

Fungicide resistance has united the industry because resistance to one fungicide typically affects fungicides with the same MOA regardless of whether the manufacturer is the same or different. Thus, it is in the interests of all fungicide companies, as well as farmers and consumers, that the efficacy of fungicides is protected for as long a period as is possible in all markets. Hence the industry has united to form the FRAC

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Table 11.1. Instances of fungicide resistance from field isolates.

Group	Mode of action[a]	Fungicide common name (example)	Risk level; high or medium or low (current assessment)[b,C]	Years between introduction and emergence of field resistance[c]	Comments
A1	RNA polymerase	Metalaxyl	H	2	Resistance is common in various oomycetes. No target gene yet detected
A2	Adenosine deaminase	Bupirimate Ethirimol	M	2	Sporadic reports of resistance
A3	DNA/RNA synthesis	Hymexazole			Resistance in FUSASO reported
B1	β-Tubulin assembly in mitosis	Benomyl Carbendazim Thiabendazole Thiophanate	H	2	Resistance common; associated with target site mutations in β-tubulin gene: E198A,G,K and F200Y. No apparent fitness penalty. High resistance factors (RFs)
B2	β-Tubulin assembly in mitosis	Diethofencarb	H	Not known	Target site mutation in β-tubulin gene: E198K. Negative cross-resistance to MBCs
B6	Actin/myosin/fimbrin	Metrafenone	M to H	10	Sporadic reports of resistance
C2	Succinate dehydrogenase inhibitors (SDHIs)	Carboxin Bixafen Boscalid	M to H	3	Several target site mutations known; cross-resistance observed. Apparent fitness penalty. Medium RFs
C3	Cytochrome bc1 quinone outside inhibitors (QoIs)	Azoxystrobin Pyraclostrobin	H	2	Target site mutations *Cytb* G143A and F129L. Cross-resistance. High RFs for G143A. Intron at 143 protects against resistance
C7	ATP transport	Silthiofam	H	10	Field resistance reported
D1	Methionine synthase (?)	Cyprodinil	M	5	Multiple MOAs linked to mitochondrial function
E1	Signal transduction (?)	Quinoxyfen	M	4	No resistance reported
E2	Osmotic signal transduction os-2	Fludioxinil	M	18	Resistance reported in BOTRCI; MDR
E3	Osmotic signal transduction os-1	Iprodione Procymidone	M	5	Resistance reported via target site in os-1 (I365N/R/S and Q369H/P) and MDR mutations. Partial cross-resistance
F9	Oxysterol-binding protein	Oxathiapiprolin	M		

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G1	C-14 demethylase in sterol biosynthesis	Prochloraz Fluquinconazole Metconazole Propiconazole Tebuconazole Tetraconazole Prothioconazole	M to H	7	Resistance is common with many combinations of mutations in *Cyp51A* and *B* gene(s), promoter mutations in *Cyp51s*, gene duplications, efflux pump overexpression especially in BOTRCI and SEPTTR. Moderate RFs. Cross-resistance moderate to high within DMIs; variable and sometimes negative
G2	Δ^{14}-reductase	Fenpropidin Spiroxamine	M		No resistance reported
G3	Keto reductase	Fenhexamid Fenpyrzamine	M	12	Resistance reported for fenhexamid. Fitness penalty suspected
H5	Carboxylic acid amides (CAAs)	Dimethomorph	H	2	Target site mutations known in *CesA8* genes: G1105A/V/S/W. Partial cross-resistance
I2	Melanin biosynthesis inhibitors –dehydratase (MBI-D)	Carpropamid	M	6	Field resistance known
U06		Cyflufenamid			Field resistance known
U27		Cymoxanil			Field resistance known

[a]Proposed MOA.
[b]H, high; M, medium; L, low.
[c]Data from Brent and Hollomon (2007a,b).

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(Fungicide Resistance Action Committee), which collates information, decides policies and dispenses advice (https://www.frac.info/, accessed 28 January 2022). FRAC operates via a global network of offices covering North America, Brazil, Argentina, South Africa, Australia, South-East Asia, China and Spain (Fig. 11.1). The main site in Europe houses committees that oversee each of the major MOA groups affected by resistance.

The economic impact of fungicide resistance can be severe for growers as well as fungicide manufacturers. A well-documented example concerns barley powdery mildew grown in Western Australia (WA). Barley is widely grown in the WA grain belt primarily for export. Substantially higher prices are obtained when the grain meets the exacting requirements of maltsters, so breeders have always focused on malting quality characters. By around 1995 a set of cultivars with good malt but poor disease resistance dominated the growing region. In response to the growing incidence of diseases such as PYRNTE and ERYSGH, seed and foliar fungicides were registered for the first time and widely used. Registrations were dominated by cheap, out-of-patent DMI fungicides, mainly tebuconazole, flutriafol, triadimefon and propiconazole. By 2011, 170 different formulations had been registered. The great majority were solo DMIs, a few were mixtures of two DMIs and only one did not contain a DMI. For about a decade, losses to these diseases were kept in check but by 2005 reports of fungicide failure became more frequent and widespread, leading to yet more frequent and widespread DMI use. As a result, mutant strains of first ERYSGH and then PYRNTE emerged. By 2011, 90% of ERYSGH isolates carried two mutations conferring strong resistance to DMIs. The resulting losses were estimated at $100 million per annum or about $200/ha not just because of the cost of fungicide application and the reduced yield, but also because the harvested grain failed to make the grade for malting and thus attracted a substantially lower price. Since 2014, new fungicides from different MOAs and new cultivars with better resistance at least to ERYSGH have been introduced. The area sown to barley has increased from 1.3 million to 1.95 million ha and the total production has gone from 2 million to 5.1 million t. Much of these

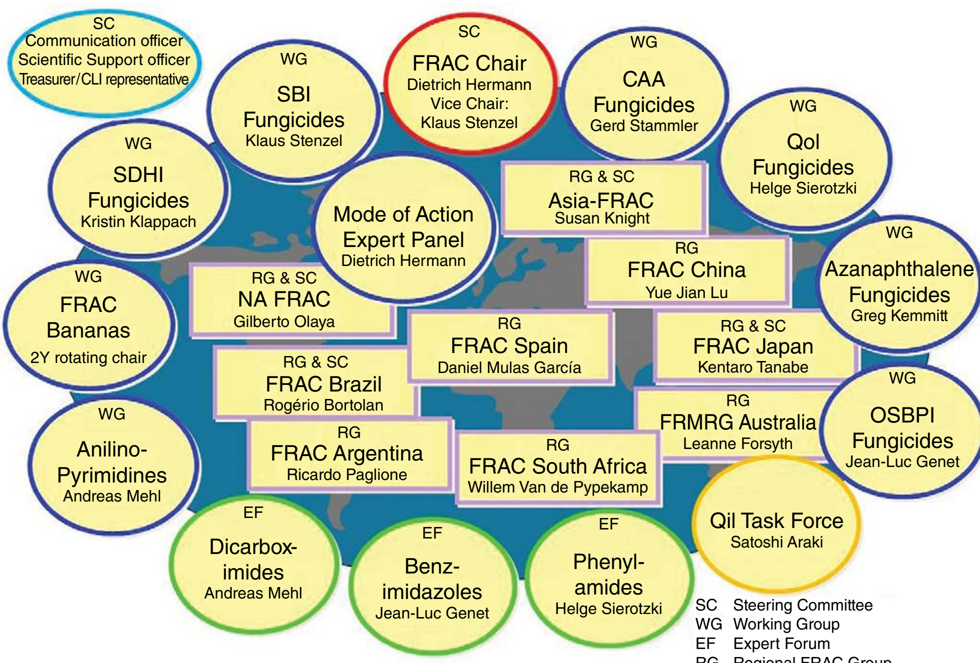

Fig. 11.1. The Fungicide Resistance Action Committee structure in 2019, comprising both regional and MOA subgroups. (From FRAC with permission.)

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increases are due to greater farmer confidence that ERYSGH can now be controlled even if PYRNTE remains a significant challenge (Tucker *et al.*, 2015, 2020; Mair *et al.*, 2019).

Fungicide Resistance Evolution

Fungicide resistance is a classic case of Darwinian evolution by natural selection. Evolution occurs when a variable population encounters a selective pressure, in this case a pressure exerted by one or more fungicides. Strains that are resistant to the selection pressure are fitter – that is, they produce more viable spores more quickly – and are therefore selected. Fungicides work by killing or slowing the growth and reproduction of pathogens and thus represent potent selection pressures. We are taught that evolution occurs over millions or billions of years, but pathogen populations can often evolve very quickly, in a matter of weeks or months. First, they have huge population sizes – a typical infected field can produce of the order of 10^{10} spores/ha. Second, they have highly variable genomes due to both conventional and fungal-specific mutagenic properties (see 'Genomics and genetic variability in plant pathogens' section in Chapter 2, this volume). Third, they often have short life cycles and can reproduce in a week or less in some cases. Fourth, many pathogen species can genetically recombine fungicide resistance genes through sexual reproduction. Lastly, they can move long distances on crop products, via rain splash or as air-borne spores. For all these reasons, the evolution of resistance can often be measured on a week-by-week basis (Oliver, 2012; Grimmer *et al.*, 2015; Mikaberidze *et al.*, 2016; Hawkins *et al.*, 2018).

Many cases of fungicide resistance have been linked to changes in the sequence of the target site protein. Furthermore, we often observe that resistance is conferred by identical mutations in different species. A good example is the G143A cytochrome b mutation found in the great majority of cases of QoI resistance. This does not mean that the fungicide directly caused this mutation. Instead, it means that the fungicide repeatedly selects the same mutations from the plethora of possibilities. All positions in a genome are (more or less) equally likely to be mutated; it is only the mutations that combine reduced sensitivity to the fungicide with continued protein function that are found in the evolved, resistant populations. A system for describing mutations in the same position in different fungi is described in Box 11.1.

Darwinian evolution does not have foresight. We frequently observe that resistant strains have several different mutations each of which contributes to the degree of fungicide resistance. This is particularly the case for *Cyp51* mutations conferring resistance to DMI fungicides. It is extremely unlikely any of these mutations occurred simultaneously. It is much more likely the mutations accumulated one after another in a stepwise fashion. Each mutation in turn must confer a sufficient selective advantage under the existing fungicide regime so that its frequency within the population increases. When the first mutation has accumulated to a sufficient degree, the fungicide regime can then select the second and subsequent mutations. It may well be that each mutational step confers only a small degree of resistance which may well be undetectable in the field and even in laboratory phenotypic tests. None the less, the selective pressure, if maintained consistently, can cause a gradual drift towards field failure.

Definitions

The fungicide resistance literature has a confusing vocabulary. The various terms used often have different meanings for farmers, agronomists, fungicide companies and fungicide resistance chemists and biologists. As in all areas of science, it is important to be clear what various terms mean.

Resistance, tolerance and sensitivity

Resistance is a term that is used to describe *genetically determined alterations in the sensitivity of a pathogen isolate to a fungicide*. We include the word 'alteration' to differentiate this from intrinsic resistance. There are many cases where wild-type isolates of a pathogen are insensitive to a fungicide – this is intrinsic resistance. For example, oomycete fungi are resistant to DMI

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Box 11.1. Nomenclature for mutations.

A standard nomenclature has been developed that allows researchers to describe nucleotide and amino acid sequence changes quickly and precisely in genes. Both systems refer to the number in the gene sequence. This can be confusing as homologous amino acids in different species can have different numbers because of different gene lengths (indels). For example, the SEPTTR CYP51 amino acid 524 is the homologue (i.e. has the same function) and presumed orthologue (derived from the same amino acid in the common ancestor of both species) of the ERYSGH amino acid 509.

Changes at the DNA level use the > sign. So 12T>A means the thymine at position 12 is replaced with an adenine. Occasionally lower-case a, t, c and g are used in the form t12a. For amino acids, the one-letter amino acid code is used. Changes at the amino acid level are in the form wild-type amino acid–number–new amino acid. An example would be the CYP51 D134G. Here, the aspartate (D) at position 134 is changed to glycine (G). If the amino acid is changed to several different amino acids in different strains, the form would be H272Y,R,L. If the amino acid was deleted, this is designated ΔY459; if two amino acids, this is ΔY459/G460. Insertions are designated Ins. So W4_R5insK means that a lysine (K) was inserted after a tryptophan (W) at position 4. Frame shifts are designated with fs. So W4fsX8 means that an insertion in codon 4 causes a frame shift at codon 8. Introduction of a stop codon, X, at a position 189 (e.g. G189X) would delete the entire C terminus of the protein from that point.

Amino acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Deletion	del	Δ
Stop codon		X
Frame shift		fs
Insertion		ins

fungicides because they lack ergosterol. Such resistance is a property of the species and a facet of the spectrum of the fungicide and should be distinguished from evolved or acquired resistance.

Where resistance has evolved, the extent of the alteration can vary from barely detectable to so large that the fungicide is entirely useless. Therefore, *resistance* encompasses a range of observations that might equate to no practical impact, to tolerance or to substantial resistance under field conditions. *Sensitivity* is the converse of resistance. *Tolerance* is in principle equivalent to resistance but is often taken to mean a low degree of resistance. The resistance or sensitivity of the fungal strain to fungicides constitutes its *phenotype*.

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Resistance occurs when the reduced sensitivity is heritable and stable. The genetic basis of resistance is a heritable alteration in a gene, such as the target, the promoter of the target gene or a transporter gene. Such heritable alterations can be detected by various forms of genetic analysis and constitute the *genotype* of the resistant strain. We must also consider *epigenetic resistance*, defined as 'a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence' (Berger *et al*., 2009), that includes modifications of histones and of DNA methylation.

Measuring resistance

There are several ways of detecting and measuring resistance. A quick but qualitative test is to grow fungal isolates (either mycelial plug or spores) on a concentration of fungicide that comfortably controls wild-type strains. This dose is known as the 'discriminatory dose' (DD) (Fig. 11.2). Strains that can grow on the DD are said to be resistant. This is a quick and cheap test as it only requires a single test dose. A slightly more refined test can use two or more concentrations of a fungicide. Strains can then be divided into sensitive, moderately resistant (tolerant) and very resistant.

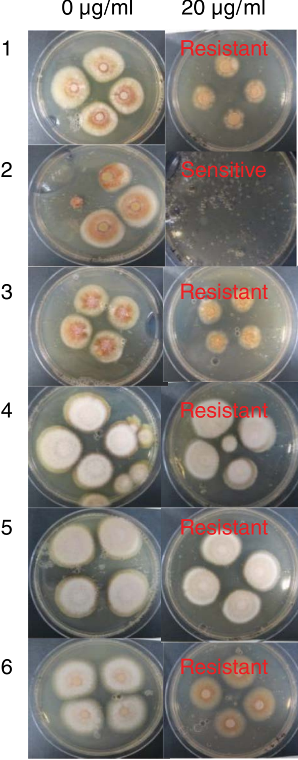

Fig. 11.2. A discriminatory dose test for six strains of *Ascochyta lentis* using thiabendazole at 20 μg/ml for 7 days. Strain 2 was classified as sensitive while the other strains were classified as resistant.

A more quantitative definition of resistance or sensitivity is the concentration of a fungicide required to inhibit growth to 50% of the level achieved in the absence of the fungicide – this is called the half maximal effective concentration EC_{50} or the inhibitory concentration IC_{50}. EC_{50} and IC_{50} are in practice interchangeable (see Box 4.1 in Chapter 4, this volume). EC_{50} values apply to one strain rather than an entire species. The EC_{50} values of a range of isolates of a range of pathogens are important baseline data that are required for fungicide testing and should be obtained before a fungicide is introduced into new regions. If these data are obtained, it is much easier to detect shifts in fungicide performance to determine whether resistance is emerging.

Non-obligate fungi can be tested in *in vivo* growth measurements. These can take the form of radial growth assays in which agar plates (see Fig. 4.2) with increasing concentrations of fungicide are prepared. The fungus is inoculated into the centre of the plates, the plates are incubated for some days and the diameter measured when the control plate is close to the boundary. The data are plotted and the concentration at which 50% growth inhibition occurs is calculated. Radial growth assays are easy and simple and do not require the fungus to sporulate, but take a good deal of time, material and space.

More precise and higher-throughput assays can be achieved using microtitre plates. In these, 96 wells can be used to test one to 96 isolates at one to 96 concentrations of fungicide. The design of the assay is very flexible. Growth of the fungi is measured by turbidometric (light scattering) measurements using a microplate reader. Large amounts of data can be acquired directly to a computer. The EC_{50} calculations can be automated, and the data stored for future use. Microplate assays work best for species that grow as yeasts; SEPTTR is a rare example. Assays with species

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that grow as filaments are less satisfactory. In these cases, a better alternative is to use a colorimetric assay based on the reduction of resazurin to a pink compound that occurs when the fungus is actively growing (Cox *et al.*, 2009).

Obligate pathogens must be tested in *in planta* assays in which a range of fungicides is applied and the degree of fungal growth assessed in an appropriate way. Figure 11.3 illustrates such an assay for ERYSGH and tebuconazole. These assays are the most requiring of time, space and material.

Ultimately, all heritable alterations in the sensitivity of a pathogen strain to a fungicide are related to a change (including an epigenetic change) in the genome sequence of the pathogen. The detection, quantification and classification of genotypic changes in pathogen populations have become major preoccupations of fungicide resistance research, not least because developments in genomics have made the analysis of genotype quicker and cheaper and applicable to larger numbers than are phenotypic methods.

Many cases of fungicide resistance are due to changes in the amino acid sequences of the target protein. The convention for describing these amino acid changes utilizes the one-letter amino acid code (Box 11.1) of the original and mutant amino acid, and the number of the amino acid. A well-known example is the E198A mutation found in the β-tubulin gene of many fungi resistant to benzimidazole fungicides. In this case a glutamate (E) residue at position 198 was replaced by an alanine (A). As the β-tubulin gene is very well conserved in all fungal species, the affected E is at position 198 in all cases.

However, for other fungicide classes such at DMIs and SDHIs, the target site proteins are less well conserved. Resistance in different species has been observed to result from the changes in the same (or closely related) amino acid (i.e. amino acid positions that are homologous) but which have different numbers due to differing lengths of the proteins. For example, a mutation in the B subunit first discovered in PYRNTE giving resistance to SDHIs is designated SDH-B H277Y

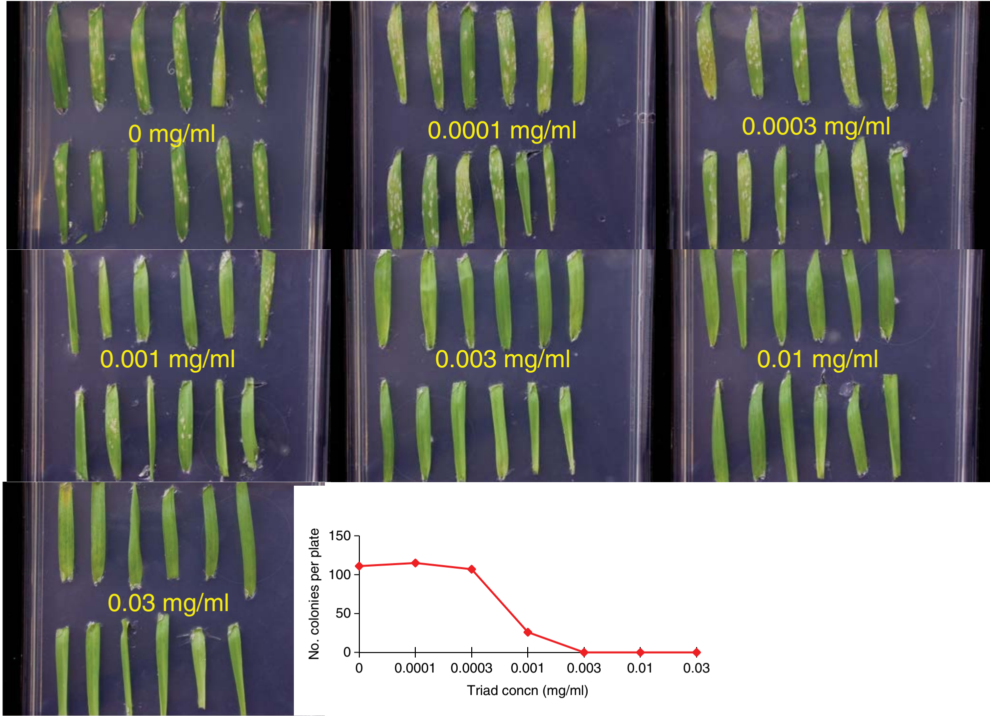

Fig. 11.3. Barley leaves infected with a single spore-derived isolate of barley powdery mildew were placed on benzimidazole agar amended with increasing concentrations of triademifon (Triad). The half-maximal effective concentration EC_{50} is estimated to be close to 0.001 mg/ml.

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but corresponds to amino acids at positions ranging from 242 to 278 in different species. To minimize confusion, Mair and co-workers (Mair *et al.*, 2016; Lopez-Ruiz *et al.*, 2017) have published a database to unify the nomenclature of genotypic changes by linking changes in all species to the orthologous changes compared with a defined archetype species. In the case of the SDH-B mutation H277Y, the orthologous changes in other species are given the italicized label *H277Y* even if the original description of the mutation in for example UNCINE was H242Y. This system will be used herein.

Resistance factor

The resistance factor (RF) is the *ratio of the EC_{50} of an isolate with evolved resistance to that of an apparently normal or sensitive isolate*. It is a useful shorthand to describe quantitatively the degree of resistance of an isolate. Isolates of a pathogen vary in myriad properties and so EC_{50} values will vary between isolates from an entirely sensitive or naïve (i.e. one that has not been exposed to the fungicide) population. Such variation can be a factor of 10, or even 100, but would vary between an EC_{50} in the range of 10–1000 ng/ml for a useful fungicide. Hence a meaningful RF can be either between two isogenic strains of the same species or, more usually, between the EC_{50} of a suspect strain and the average EC_{50} of a set of naïve strains.

RFs can be divided arbitrarily into low (<10), moderate (10–50) and high (>50). Higher RFs occur when the mutation giving the resistance gives a very high level of resistance. Low or moderate RFs are often termed tolerance or reduced sensitivity rather than resistance. There is no clear-cut value at which an RF can be said to be high enough to be significant in the field. Clearly very high RFs (say >100) will normally mean that the maximum field rate of the fungicide has no useful or even discernible impact on disease levels in the field. However moderate RFs may well have an impact on the evolution of the population, eliminating the sensitive isolates as the weakly resistant one takes over. RFs as low as 1.5 have been seen to have this effect. Continued use of the same fungicide year after year may lead to a stepwise loss of efficacy which ultimately has major field significance, as was seen with DMI fungicides.

Field resistance

The term ‘field resistance’ has two distinct usages. One usage refers to an observation that the efficacy of the fungicide in a field-grown crop is substantially reduced. This usage contrasts with resistance that is of such low impact that the RF can only be detected using laboratory tests and has little or no obvious impact in a field setting. The second definition refers to the source of the resistant isolates. Field resistant isolates arose naturally in the field and presumably are fit enough to survive. In contrast, laboratory resistant isolates have been selected following some sort of laboratory procedure. The first type of field resistance is what really matters to a farmer. Its occurrence depends on two factors:

1. Whether the EC_{50} of the resistant strain is high enough to protect the fungus against the field rate of the fungicide.
2. Whether the prevalence of the resistant strain is high enough to enable it to dominate the population.

Cross-resistance

Cross-resistance is the phenomenon when a *strain resistant to one fungicide is found to be altered in resistance to another fungicide*. The two fungicides are then said to exhibit cross-resistance. Cross-resistance is a quantitative parameter. In some cases, the RF with one fungicide is similar to another. This is typically the case within the QoI and MBC fungicide classes. RFs to QoI fungicides of G143A strains may vary from 40 to 100 but they are all high and thus all QoI fungicides are rendered practically useless when resistance to one has evolved. Partial cross-resistance applies when the RF with one fungicide is much lower than with another. This is the case within DMI fungicides where RFs vary between 1 and 20. In these cases, the fungi may show high resistance to one DMI but be relatively sensitive to another.

Cross-resistance is normally described as positive; that is, the resistant strain is more

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resistant to both fungicides. Or to put it mathematically, both RFs are >1. There are a few cases of negative cross-resistance. Here the strain resistant to one fungicide is more sensitive to another fungicide than the wild type; that is, one RF is >1 and the other is <1. This can occur when mutations in the target site gene alter the physical conformation of the target site. Negative cross-resistance can occur if the mutated target site binds the second fungicide less tightly than does the wild-type target site. It has been observed in fungicides that target β-tubulin and the *Cyp51* gene. This concept of negative cross-resistance suggests a cunning strategy to combat fungicide resistance, in which the two fungicides are used sequentially. The first spray selects for pathogen strains that are particularly sensitive to the second fungicide. The second fungicide efficiently controls the population but eventually resistance will occur again. These strains are now sensitive to the first fungicide. In principle we could alternate between these fungicides so that disease levels were always acceptable. Attractive though these schemes might appear to be, there are no cases where negative cross-resistance has been used as a deliberate resistance management strategy.

Most cases of cross-resistance involve fungicides from the same MOA and there has often been critical evidence identifying and linking the MOAs of different fungicides. This was the case with the CAA fungicides (Blum *et al.*, 2011). Cross-resistance typically involves mutations in the coding region of the target site gene or mutations that increase the concentration of the target site protein in the fungal cell, mainly insertions in the promoter that increase gene expression or duplications of the entire gene.

Multiple resistance; multi single-, oligo- and multi-drug resistance

We are increasingly finding strains of fungi that display a phenotype whereby they are resistant to fungicides from several different MOA classes. The record number appears to be seven for various BOTRCI populations (Fernández-Ortuño *et al.*, 2015; Weber and Entrop, 2016; Samarakoon *et al.*, 2017). There are two ways that fungi can be resistant to more than one class of fungicide. The first mechanism is where fungal strains have acquired many target site mutations each giving resistance to a single fungicide group. This phenomenon has been called *multi single-drug resistance* (MSDR) but is perhaps better called *oligo-drug resistance* (ODR) to distinguish it from MDR. The second mechanism is *multi-drug resistance* (MDR) and is caused by increased activity of efflux pumps that remove fungicides from the cytoplasm of pathogen cells. Such pumps can reverse the inflow of multiple different classes of fungicides from the exterior medium and thereby decrease the intracellular concentration.

Metabolic resistance

Resistance to herbicides is also a significant problem for the crop protection industry. Unlike fungicides, where target site modes of resistance (MORs) dominate, the commonest cause of herbicide resistance is termed metabolic resistance (Yu and Powles, 2014). Resistant weed populations show enhanced expression of genes encoding enzymes such as cytochrome P450 monooxygenase, glycosyl transferase and glutathione *S*-transferase. These enzymes modify the herbicide by oxidation and conjugation so that the herbicide is rendered inactive or can be transported to a vacuole or outside the cell and away from the target site. Metabolic resistance often affects herbicides of different chemical and MOA groups. Until recently no case of metabolic resistance had been observed in plant–pathogen interactions. Recently, however, reports of metabolic resistance in the dollar spot turfgrass pathogen (SCLEHO) have appeared (Green *et al.*, 2018; Sang *et al.*, 2019).

Fitness penalty

Fungicides select for mutations in the pathogen population that confer a selective advantage on the strain in the presence of the fungicide. The selective advantage may be expressed as a high EC_{50} and hence a large RF. If the mutation is significant in the field, the proportion of the pathogen population that carries the mutation will increase until it dominates the population from season to season. Such strains are said to

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carry a fitness advantage in the presence of the fungicide. The term 'fitness' is used in the evolutionary sense: 'survival of the fittest', and thus applies to overall ability to reproduce and cause disease from year to year.

A very important question is whether the mutant strain is as 'fit' (i.e. grows as fast and produces as many viable spores) as the wild-type sensitive population in the *absence* of the specific fungicide class. If the mutant population is less fit than the wild type in the absence of the fungicide, the resistant strain is said to carry a *fitness penalty* (Hawkins and Fraaije, 2018). There are many reasons why a resistant population might carry a fitness penalty, and not all fungicide resistant isolates suffer from such a penalty (Chapman *et al.*, 2011). It may be that the target site mutation which confers resistance has the side-effect of reducing the efficiency of the enzyme. This appears to be the case for DMI and SDHI fungicides. In the case of MDR caused by efflux pump resistance, it may be that the metabolic energy required to synthesize and drive the pumps represents a significant drain on the energy resources of the pathogen.

If the fitness penalty is substantial, removal of the fungicide should allow the re-emergence of the sensitive population of the pathogen. In this case, the previously compromised fungicide could then be usefully deployed again, for a while at least. And (it is hoped) better fungicide resistance management strategies can be applied.

It is fair to say that no simple, broadly applicable and meaningful methodology to measure fitness penalty has yet been developed. The term is applied to growth rates of fungi in artificial media (without fungicides) and more rarely to the growth rate of symptoms in growth chamber, glasshouse or field trial experiments. A promising strategy is to co-inoculate two strains and measure the relative prevalence of the strains after one or more infection cycles. It is worth noting that a fitness penalty of 10% per annum would mean something like a decade without use of the fungicide would be needed for a sensitive strain to replace the resistant strain.

Resistance Risk

The risk that resistance will develop is clearly an important parameter. It defines the sustainability of the fungicide product over several seasons. Resistance risk is affected by the properties of the pathogen, the fungicide class and the way the fungicide is used in the field.

Pathogen risk factors; fecundity; latent period; sexual reproduction

Fungicides that are mutagenic would not proceed to the marketplace. Stringent tests are applied to fungicides to ensure that they have no mutagenicity. Instead, fungicides merely select strains that have enhanced resistance by enforcing an evolutionary selection pressure (Hobbelen *et al.*, 2014). Even when diseases are well controlled their population size can still be huge, comparable with the size of typical pathogen genomes. Pathogens typically have genome sizes of 40 million to 100 million base pairs and express 6000 to 15,000 genes. Normal processes of spontaneous mutation caused by UV or other radiation, by environmental chemicals and by failures of DNA replication repair processes would be expected to generate changes in 1×10^{-6} genes and 1×10^{-9} base pairs per nuclear generation. Thus if 10^9 spores are produced after 10–100 mitotic cycles in a pathogen population, most base pairs in the genome would be altered in at least one spore that is present. It has been estimated that 100 m^2 of barley moderately infected with powdery mildew would have a 95% chance of containing strains carrying all of the viable mutations (Brent and Hollomon, 2007a). It therefore is apparent that pathogens that produce large numbers of spores are at a higher risk of developing resistance than those that produce fewer spores. Also infections that are poorly controlled by either genetics or fungicides will produce more spores than if the disease is well controlled (see 'Genomics and genetic variability in plant pathogens' section in Chapter 2, this volume, for a discussion of mutagenesis in pathogens). This is referred to as the emergent phase of resistance (Hobbelen *et al.*, 2014; Mikaberidze *et al.*, 2017).

When fungicide-resistant strains are present in the population and there is therefore a mixture of the mutant strain and the wild type, normal evolutionary selection processes come into play. When this mixed population is exposed

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to the fungicide, a higher proportion of the wild-type strain will be killed whereas a higher frequency of the mutant population will survive and reproduce. In other words, the resistant population has a higher growth rate than the sensitive. The proportion of the population that is resistant will increase each time the fungicide is applied. For the first few cycles of selection, the frequency of the resistant population is unlikely to be noticeable. However, if the pathogen population reproduces frequently and the fungicide is reapplied, then the selection can be applied time and again and the resistant population can increase in frequency until it comes to dominate the population. If the RF is also high enough, the result then is field resistance. Thus, pathogen species that reproduce multiple times within a season are higher risk. Conversely, pathogens with long latent periods are low risk. Seed-borne pathogens that only have a single life cycle per season are low risk. In contrast, pathogens that have short life cycles and can infect for an extended period of the growing season are high risk (Fig. 11.4).

Distribution of spores is also a significant factor. Pathogens that can spread far and wide are clearly a higher risk of generating significant fungicide resistance epidemics than ones that move short distances. Species with wind-borne spores are therefore considered high risk, rain-splashed spores are intermediate, and water-borne and soil pathogens are the lowest risk (Brent and Hollomon, 2007a).

Some cases of fungicide resistance involve mutations in more than one gene. These could be genes for resistance to two different MOA

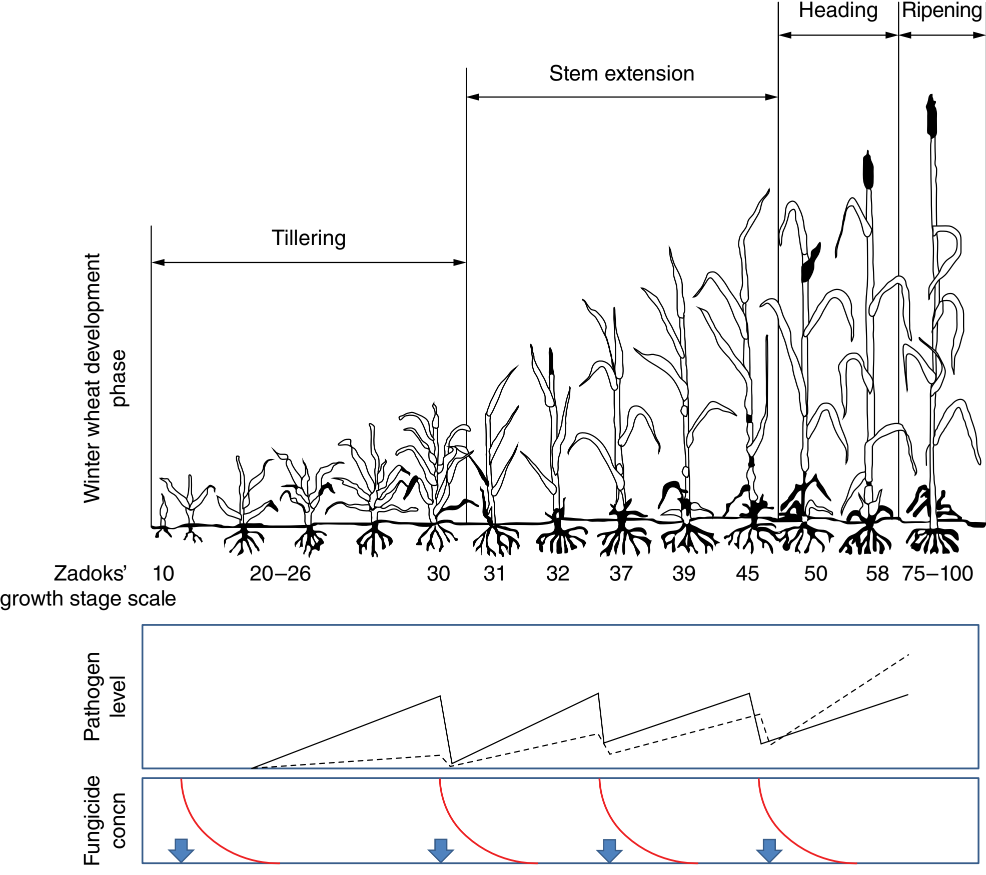

Fig. 11.4. A polycyclic pathogen with a short life cycle controlled by multiple fungicide sprays (fungicide applications arrowed) is at high risk of resistance evolution (– – –, resistant; ——, susceptible).

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classes or two mutations that had an additive effect on the resistance to one MOA class. In both these cases, combinations of genes would be much more of a threat than the single mutations. Pathogen species that are able to undergo sexual reproduction and hence recombination therefore are more likely to evolve strains capable of combining several mutations that confer a significant selective advantage (Mikaberidze *et al*., 2017).

Based on these factors we can divide fungi into three classes: low, medium and high risk, and compare these classes with the now 30-year history of fungicide resistance. Table 11.2 summarizes relevant features of some important pathogens and their history of resistance development.

This crude analysis shows that, by and large, the theoretical prediction has been borne out by experience. BOTRCI, powdery mildews, MYCOFI, PLASVI and PHYTIN have consistently been the first species to display resistance to fungicides. One unexpected exception is the cereal rusts, which have many of the characteristics of high-risk pathogens – large population sizes, air-borne spores, short life cycles, sexual reproduction – but have so far failed to display significant resistance. However, this is not true for the closely related species PHAKPA which has displayed resistance to DMIs, SDHIs and a weak form of QoI resistance in South America. The apparent immunity of cereal rusts to fungicides appears to be due to a combination of factors which are specific for each class of fungicide (Oliver, 2014). Rust fungi are not sensitive to five major classes of fungicide to which resistance has evolved: A1, B1, D1, E2 and E3 (see Table 11.1), and the early SDHIs like carboxin were not used on foliar diseases. This leaves just three major single-site fungicide classes currently used to control cereal rusts.

For QoI fungicides, rust species carry the 'blessed intron' which renders the G143A mutation inviable (Grasso *et al*., 2006). PHAKPA strains carrying the weaker F129L allele have evolved (Klosowski *et al*., 2016) and this can be expected to occur in cereal rusts. DMIs are widely used to control rusts and a few hints of resistance have been reported in cereal and soybean rusts (Stammler *et al*., 2009; Schmitz *et al*., 2014; Reis *et al*., 2015; Cook *et al*., 2021; De Mello *et al*., 2021). The third class is the newer SDHI fungicides suitable for foliar application and with broad spectrum. PHAKPA isolates with resistance to these SDHI fungicides have recently been detected (Simões *et al*., 2018; De Mello *et al*., 2021). It therefore appears that rust species should be regarded (at best) as a medium risk for resistance and it would be prudent to remain vigilant.

Table 11.2. Fungicide resistance risk factors and risk level classification for selected pathogens. (From Brent and Hollomon, 2007a and FRAC, with permission.)

Pathogen	Fecundity	Latent periods	Sexual reproduction	Resistance prediction[a]	Resistance history[b]
BOTRCI	High	Many	No	High	High
Powdery mildews	High	Many	Yes	High	High
PYRIOR	High	Many	No	High	Medium
PHYTIN	High	Many	Yes	High	High
VENTIN	Medium	Medium	Yes	High	High
PLASVI	High	Medium	Yes	High	High
RAMUCC	Medium	Medium	No	High	Med
MYCOFI	High	Medium	No	Medium	High
SEPTTR	Medium	Medium	Yes	Medium	High
PSDCHE	Low	Medium	Yes	Medium	Medium
RHIZSO	Low	Long	No	Low	Low
Soil-borne pathogens	Low	Long	No	Low	Low
Seed-borne pathogens	Low	Long	Some	Low	Low
Rusts	High	Short	Yes (some)	Low	Medium

[a]According to Brent and Hollomon (2007a).
[b]Authors' summary of current data.

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Table 11.3 lists the pathogens that should now be regarded as being at high risk of resistance. All of these have several published cases of field-relevant resistance. Two of these newly recognized high-risk pathogens, SEPTTR and PHAKPA, are now utilized as key primary test organisms in fungicide discovery. The phylogenetic spread of high-risk organisms has widened to include a rust. The question arises as to whether other rusts and especially the cereal rusts should be considered high risk. Research into cereal rust control focuses mainly on host genetics but major epidemics of brown rust (Ug99) in Africa and South Asia and of yellow rust in Europe and Australia have placed unprecedented pressure on fungicide selection. Rusts all harbour the *Cytb* G143 intron and this explains why QoI resistance has been so far undetected other than via F129L in PHAKPA. Because of the development of resistance to DMIs in other cereal pathogens, rusts are much more regularly exposed to SDHI fungicides than in previous years. It would be prudent to regard cereal rusts to be at a high risk of resistance to SDHIs and to put in place suitable monitoring systems such as provided by genomic methods (Hubbard *et al*., 2015; Cook *et al*., 2021).

Fungicide risk factors

History has demonstrated that the risk of resistance differs markedly between fungicide groups. Table 11.1 gives the time in years between the introduction of a fungicide and the emergence of field resistance. Some fungicides have never developed significant resistance whereas others have developed resistance in as short a period as 2 years. Some cases of resistance occur wherever and whenever a fungicide is used, while in other cases resistance is rare and sporadic. Understanding the reasons behind these differences has become a major goal of the fungicide industry because it might allow the design of fungicides with a lower risk of resistance or prolong the useful life of a fungicide.

It is essential, for both commercial and regulatory reasons, to estimate the risk of resistance before a fungicide is released into the field. This is especially true when the fungicide has a new or even unknown MOA. We want to determine whether any resistant strains can be produced, how common they are and whether any fitness penalty exists. One approach is experimental mutagenesis and selection *in vivo*. In this scenario, a large population of a test fungus is treated with the fungicide to determine whether any spontaneous resistant mutants can be detected. To reduce the size of the population that needs to be tested, the fungus can be treated with a mutagen such as UV or gamma rays, sodium azide or ethylmethanesulfonate. Model fungi such as *Saccharomyces* or *Neurospora* are often used for this purpose because these species are easy to handle in the laboratory and have well-developed genetic resources that can be used to determine the genetic basis of resistance and the MOR. Other high-risk fungi such as BOTRCI and PHYTIN are also used. Despite the

Table 11.3. High-risk pathogens. (Authors' own data.)

Pathogen	Host	Disease
ALTESO	Tomato	Early blight
CERCBE	Sugarbeet	Cercospora leaf blotch
UNCINE	Grapevine	Powdery mildew
PYRIOR	Wheat	Blast
PENIDI	Citrus	Postharvest
PHAKPA	Soybean	Asian soybean rust
PHYTCP	Cucurbits and *Solanaceae*	Blights and rots
PHYTMS	Soybean	Stem and root rot
PYRPBR	Oilseed rape	Light leaf spot
PYRNTE	Barley	Net blotch
SCLEHO	Turfgrass	Dollar spot
SCLESC	Oilseed rape	Stem rot
USTNVI	Rice	False smut
SEPTTR	Wheat	Septoria tritici blotch

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technical difficulties even powdery mildews have been tested.

Laboratory mutants have been found for many fungicides and species (see FRAC, 2019) and have proved to be of great value in the determination of the MOR. In a number of cases, equivalent field mutants have not so far been found. In other cases, field mutants have been found but the genotype of mutants differs from that found in the laboratory. The successful recovery of laboratory mutants indicates the potential for that species–fungicide combination to develop resistance in the field. Failure to find field mutants resistant to the fungicide can arise from two factors. First, it may be that the fungicide has not been applied to a large enough area over a long enough time for resistance mutants to develop. Second, it may be that the resistant mutants carry a substantial fitness penalty that such strains die out when grown under field conditions. This may also explain why different genotypes are found in the field and the laboratory. The converse situation, where field resistance has been found but laboratory mutants could not be generated, is much rarer. In a few cases field resistance to non-systemic fungicide developed after decades of use. In these cases, the fungicide has been used for a long period over a wide area, whereas the period allowed for detection of laboratory mutants is only a few weeks at most.

Monitoring for field resistance

In the past, reports by growers of occurrences of fungicide failure were the first indications that resistance might have developed. The primary interaction was normally between the fungicide reseller and the grower. If the disease developed despite the application of the new and expensive fungicide, the grower normally wasted no time in letting the reseller know. The reseller then typically reported back to the local company representative who would then try and obtain an isolate from the affected field for analysis in the laboratory. Experience showed that the great majority of cases could not be ascribed to resistance. Much more likely were problems with the formulation batch, weather conditions, spray equipment and spray coverage.

In view of these factors and because of the supreme importance of resistance to fungicide companies, monitoring for resistance for new and existing fungicides has become a much more systematic activity. Dedicated field trials are used and intensively monitored. National organizations, such as the Agriculture and Horticulture Development Board (AHDB) in the UK, carry out these trials. Each major fungicide company carries out its own trials along these lines also, although the results are not necessarily made public immediately. The trials target high-risk pathogens and use a range of concentrations to determine the efficacy graph. The trials are repeated year on year so any declines in efficacy are apparent. In addition, many farmers' fields are inspected each year and unusual cases of disease are noted. In the UK this is called Crop Monitor (http://www.cropmonitor.co.uk/, accessed 28 January 2022). Suspect isolates from these studies can be collected and tested under controlled conditions.

If resistance is suspected, it is important to determine what proportion of the pathogen population is affected. To achieve this, the researcher needs to acquire a set of random samples of pathogen isolates. To determine the frequency of phenotypic resistance, isolates of the pathogen must be tested using DD or EC_{50} tests as described above under 'Measuring resistance'. If we want to detect resistance before it has become a significant problem, a very large number of isolates needs to be tested. If a resistance is present in 1% of a population, it is necessary to sample 300 isolates to have a 95% chance of finding one case of resistance. To determine a statistically robust estimate of resistance prevalence, many hundreds or thousands of isolates are needed from each region, crop and disease. This is a major expense for fungicide companies.

To reduce the number of isolates needed to detect the emergence of resistance, so-called 'bait tests' can be applied. A small experimental plot is set up in a susceptible crop and in the vicinity of a natural inoculum. The plot is treated such that a gradient of fungicide dose is applied ranging from a quarter dose through half, full and double dose. The researcher acquires pathogen isolates from the highest dose to show disease symptoms and these are then tested for phenotypic resistance. This reduces the number of isolates that are needed to detect resistance but can only give a qualitative estimate of the frequency of resistance.

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Phenotypic and genotypic monitoring

In the early years of use of a new fungicide, it is essential that isolates are tested using reliable and quantitative phenotypic tests for resistance. It may well be that laboratory mutants had been obtained prior to launch and that the genotypic basis of resistance has been determined. However, experience has shown that the field provides a very different environment from any laboratory test and different genotypes are often observed. Indeed, even when the genotypic basis of resistance has been studied, a subset of isolates should be tested phenotypically in case new mutations have emerged.

However phenotypic tests are expensive and laborious. By the time a fungicide is released in secondary markets, the genotypic basis of resistance may well have been determined in primary markets years or decades earlier. In such cases, genotypic tests may well be suitable. Genotypic testing methods date back 20 years or more but are undergoing rapid improvements in speed and cost. It is now feasible to collect diseased plant samples, process them in an hour or so using hand-held instruments, and deliver the grower a report on the presence and quantity of major pathogens and the frequency of a number of mutations conferring fungicide resistance (Dodhia *et al.*, 2021). Such tests can even be applied to spores before they have landed on the crop. These technologies promise to give farmers the knowledge they need to decide whether to spray and which fungicides to use.

The practical application for detection of pathogen DNA from a collection of plant samples to the results is now reported for various battery-powered devices using loop-mediated isothermal amplification (LAMP). LAMP requires the design and testing of a complex set of primer sequences for both specificity and sensitivity and hence the genome sequence of the pathogen and likely non-target contaminants must be known (Niessen, 2015). The technology can detect a known sequence with good sensitivity in plant tissue samples collected and processed in the field within an hour or less. It can also be applied to the detection of pathogens with a known fungicide resistance genotype (Duan *et al.*, 2015).

Such a capability is within reach using instrumentation such as the Luminex xTAG digital PCR (Ishii *et al.*, 2008; Kostov *et al.*, 2015). Current versions of these machines can generate up to 20,000 individual PCR reactions and have the sensitivity to detect a mutation that is present at or below 1%. The timescale with these tests is a matter of a few days and the costs associated are significant.

Digital PCR is currently able to distinguish two or three different genotypes in a single experiment. Mutations giving resistance to a fungicide often occur at many places within and outside a gene. Hence to get a fuller picture it would be desirable to generate the sequence of many isolates (nuclei) over the target gene, or indeed the entire genome. One method is to use pyrosequencing, which gives data over 300–500 base pairs and can accommodate up to 500 isolates in a single sample (Gobeil-Richard *et al.*, 2016). Pyrosequencing can thus be used to detect and quantify all the different versions of a ~500 bp sequence in a large sample.

Methods to sequence all the RNA transcripts in a fungal pathogen and host mixture have been pioneered by Hubbard *et al.* (2015) albeit without a specific focus on fungicide resistance. In that study, infected plant samples were stabilized in a proprietary fluid-containing vial, posted to the sequencing facility and the mRNA sequenced. The costs were substantial but could be reduced by focusing on specific pathogen genes. The timescale was a matter of a few weeks, but substantially less than the several months used previously to assay the rust pathotypes.

To go one stage further, it is now feasible to perform whole-genome DNA sequencing of infected plant material. This would not only detect known gene mutations associated with resistance, but also would give clues to new previously undetected mutations (Hu *et al.*, 2019; Cook *et al.*, 2021).

The field of genotypic microbial testing is moving at a dizzying pace, driven by advances in clinical applications. When costs and equipment are suitable for agricultural use, they promise to revolutionize the management of fungicide resistance.

Determining the mode of resistance

Should resistant mutants be recovered from laboratory studies or the field, they can be used to determine the MOR. This field of research has been impacted significantly by recent developments

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in genomics (Cools and Hammond-Kosack, 2013). The goal is to identify the gene(s) that have mutated and been selected to give the resistance. Basic parameters will be collected: the frequency of mutants, the EC_{50} on the test fungicide and whether cross-resistance is found to other fungicides. Cross-resistance with fungicides from different MOAs would indicate non-target site mutations. If the fungicide is related to known MOAs, the target site genes can be amplified by PCR and sequenced. Genetic analysis, crossing the mutant strain to a wild type, is possible in some fungi and was used to determine the MOR of H5 CAA fungicides (Grenville-Briggs *et al.*, 2008) and to refine the MOR of the D1 anilino-pyrimidine fungicides (Mosbach *et al.*, 2017).

The asexual or epidemic growth stage of most plant pathogens is haploid and therefore any mutational changes are expressed immediately. If the mutant is fit, its development in the population can be rapid. In contrast, the oomycetes are diploid and the basidiomycetes, such as the rusts, are dikaryotic in their pathogenic phases (i.e. it contains two nuclei and is therefore binucleate). In these cases, we need to consider whether the resistance is recessive or dominant or semi-dominant. Dominant mutations are expressed when the mutation is present in only one of the two nuclei and therefore provides immediate and selective advantage in the presence of the fungicide. Recessive mutations would need to go through a meiotic phase before the phenotype would express resistance. Semi-dominance would be intermediate between these extremes.

If the MOR is still unknown after all these analyses have been carried out, the newer genomic methods can be applied. The genome sequences of all major target pathogens have now been determined. In principle, it would therefore be a simple matter to sequence the genome of a resistant isolate and identify changes in the genome compared with the reference genome. Unfortunately, the general level of sequence variation between isolates is very high, so identifying the mutation responsible for the fungicide resistance requires further evidence. One type of further evidence is to sequence more strains, both resistant and wild type. Any sequence variations that occur between wild-type strains can be discarded but any sequence variation in common in the resistant strains and absent in the wild type will pinpoint the likely affected site. A second type of evidence is to examine gene expression into mRNA in the wild-type and mutant strains. Gene expression data can easily be obtained using RNAseq techniques. Genes that are expressed at a higher level in the mutant compared with the wild type, in the absence or especially the presence of the fungicide, will gives clues both to the MOA and the MOR. Finally, the proteome or metabolome of mutant strains can be examined. These refer to all the proteins and all the metabolites found in a biological sample. Facile methods to enumerate these 'omes are well established.

Fungicide Resistance in Different Fungicide Classes

Multi-site fungicides

Fungicides that act against several biochemical targets (multi-site inhibitors) are typically immobile, surface-acting protectants and are regarded as zero- to low-risk compounds. With few exceptions, their effectiveness has remained constant throughout many years of intensive use against a wide variety of pathogens.

Mercury fungicides were first described in the late 19th century and were used extensively as cereal seed treatments for broad-spectrum disease control. Their effectiveness against *Pyrenophora graminea*, the causal organism of barley leaf stripe, began to decline only in the 1980s, attributed to the development of resistance operating through the increased efficiency of mercury efflux from the fungus. In contrast, no resistance to copper-based fungicides has been reported even though resistance to copper toxicity has been observed in bacteria, yeasts and higher plants. This strongly suggests that the genes that govern similar resistance to copper toxicity in fungi are absent.

Fungal resistance to other multi-site inhibitors, such as the dithiocarbamates, phthalimides and sulfur, is unknown. It is postulated that a very large number of genes would need to mutate to give resistance and that fitness penalties would be too large to be tolerated. Although multi-site inhibitors are severely restricted in their commercial applications and value, their non-specific MOA has

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clear advantages over specific target-site fungicides in terms of resistance development.

Single-site fungicides

Fungicides that target a single site are substantially more prone to resistance development than multi-site fungicides. This is because it may well be that just one mutation of the gene encoding the target site protein is sufficient to achieve resistance. Whether field resistance emerges is dependent on the following factors:

- the RF associated with the resistant mutation(s) – this determines the ability of the mutant to grow and reproduce after treatment with field rates of the fungicide; and
- the presence and scale of a fitness penalty in the viability of mutant strains – at one extreme resistance mutations are lethal, in others the mutant is partially compromised, while in others there is no deleterious effect.

The MORs come in four forms (Fig. 11.5):

1. *Target site mutation* – mutations of the target site gene rendering the gene product less sensitive to the fungicide. The fungicide binds to the mutant target site less tightly than to the wild type, so a greater concentration of fungicide is needed to achieve the same level of binding and hence inhibition. Target site mutations typically affect all fungicides with the same MOA but the RFs for each fungicide may vary and even can be <1 (negative cross-resistance) if some active ingredients bind a mutant form less tightly than the wild type.

2. *Target site overexpression* – overexpression of the target site gene so that the total capacity of the target pathway is increased. Extra copies of the target protein/enzyme are produced so more fungicide is needed to achieve the same inhibitory affect. This typically affects all fungicides with the same MOA. RFs for each fungicide are positive and tend to be rather similar in value. It typically does not affect fungicides that target proteins that function as multimeric complexes with other proteins, such as MBC, SDHI and QoI fungicides. Such multimeric complexes require that the component proteins are produced in stoichiometric (normally equal) amounts. An oversupply of one protein is likely to disrupt the function of the complex and incur a large fitness penalty.

3. *Efflux pump overexpression* – upregulation of efflux pumps such that the internal concentration of the fungicide is kept below a critical level. Again, more fungicide must be applied to achieve the same level of inhibition. This affects fungicides from more than one MOA class, so is often called multi-drug resistance (MDR).

4. *Degradation* of the fungicide via glycosylation or other chemical modification. In contrast to herbicides, this MOR is not important in current fungicides and has only been reported for one pathogen (SCLEHO) (Green *et al*., 2018; Sang *et al*., 2019). It is often called *detoxification*.

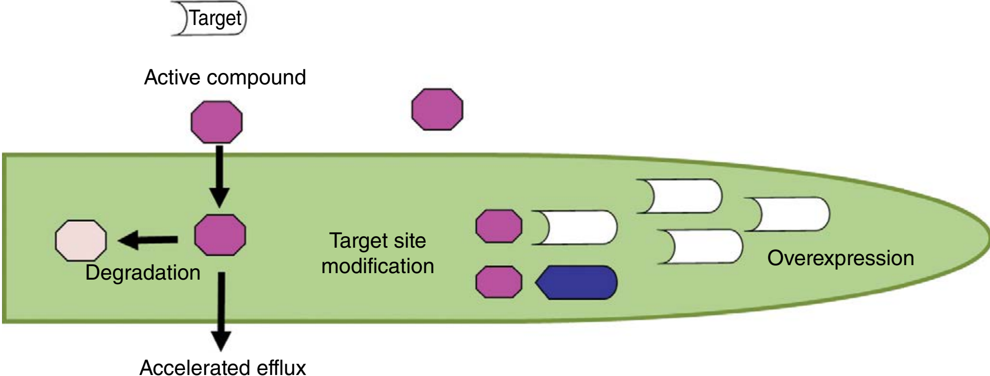

Fig. 11.5. The four classes of fungicide resistance mechanisms: target site modification, target site overexpression, efflux pump overexpression and degradation. (Authors' own figure.)

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These factors are illustrated in the following discussion of the six major fungicide classes that have been most significantly affected by resistance.

B1/2; Methyl benzimidazole carbamates

The MBCs were the first systemic fungicides to be marketed, appearing in growers' fields in 1970. Benomyl was hailed as a magic bullet and so when resistance appeared just 2 years later, it sent shock waves through the industry. MBC resistance became the prototype case of evolved fungicide resistance and helped established the sub-discipline.

Resistance first appeared in the sugarbeet pathogen CERCBE. Ideal conditions for the disease occur in northern Greece and at that time sugarbeet could not be grown without the use of fungicides. The main actives used at that time were the immobile protectant organotin compounds such as fentin acetate, but under high disease pressure and especially in sprinkler-irrigated situations such products gave inadequate levels of control. In 1967, experimental field testing of benomyl against CERCBE showed a twofold superiority in control compared with the organotin products. Support grew for the replacement of protectant fungicides with the new systemics and by 1972 more than 3000 ha were treated exclusively with benomyl.

Excellent disease control was observed during 1970 and 1971 but by July 1972, catastrophic failures were observed. Within 20 days, the proportion of infected leaves per plant increased from 5–10% to 80–100%. Increasing the application rate and frequency of application had no effect on the level of disease control. In comparison, the traditional use of organotin products, maintained in side-by-side field plots with benomyl, performed as expected (Georgopoulos and Dovas, 1973) (Table 11.4).

Table 11.4. The performance of benomyl and fentin acetate against CERCBE in northern Greece, 1970–1972. (From Dovas *et al*., 1976, used with permission.)

	Proportion of diseased foliage (%) in mid-August	
Treatment	1970	1972
Benomyl, 300 g/ha	5.9	85.9
Fentin acetate, 500 g/ha	19.3	39.6
Control	100.0	100.0

At first the loss of disease control was attributed to the weather conditions, but soon the real cause of the phenomenon was discovered to be resistance. Prior-use patterns of benomyl in 1970 and 1971 correlated with the occurrence of resistance in 1972. The high selection pressure of the benzimidazoles was demonstrated in experimental plots. A low initial disease incidence of less than 5%, caused by resistant strains of CERCBE, increased to over 90% in less than 6 weeks despite two applications of benomyl. Resistant strains were of equivalent fitness to the sensitive strains, in common with other benzimidazole-resistant fungi (Dovas *et al*., 1976).

Isolates of the fungus made from lesions in fungicide-treated plots were found to show resistance *in vivo* to benomyl. The genetic basis of the resistance was studied using the model fungus NEUSCR and shown to be a single dominant gene (Borck and Braymer, 1974). The gene was identified as that encoding β-tubulin in the yeast SACCCE (Thomas *et al*., 1985). The β-tubulin gene is highly conserved and with the advent of PCR and DNA sequencing techniques it was quickly shown that most resistant mutants in different species not only involved the same gene, but also the same small number of DNA sequence changes. The change most commonly seen is E198A but E198G,K, F200Y and several other mutations have been found (Table 11.5) (see Box 11.1 for an explanation of nomenclature rules describing sequence variations). Mutant versions of the β-tubulin protein were found to bind less tightly to the fungicides, confirming that the MOA of the fungicides was to prevent the polymerization of tubulin and thus inhibit nuclear division. The MOR was defined as reduced binding of the fungicides to the mutant β-tubulin. Expression of the mutant versions in fungal transformation experiments verified that this mutation was the primary cause of the field resistance (Cooley *et al*., 1991).

The RFs associated with these changes are very high, >100 in many cases. Indeed, the mutants are so resistant that it is hard to dissolve an inhibitory concentration of the fungicide. Resistance affects all the B1 MBC fungicides:

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Table 11.5. β-Tubulin mutation position and changes – archetype ASPEND. (Authors' own table.)

Position and change	Species
H6L/Y	LEPTNO, MONIFC
Y50N/S/C	GIBBFU, GIBBZE
F167Y	CERCBE, COCHHE, GIBBZE, NEUSCR, PENIEX
E198D/K/Q/A/V/L/G/V	BOTRCI, CERCBE, GIBBFU, GIBBZE, HELMSO, MONIFC, PENIAU, PENIEX, PENIIT, PYRPBR, RHYNSE, SCLEHO, SCLESC, SEPTTR, VENTIN
F200Y	BOTRCI, GIBBFU, GIBBZE, PENIAU, PENIIT, RHYNSE, VENTIN
L240F	MONILA, PYRPBR, VENTIN

carbendazim, thiabendazole, fuberidazole, thiophanate-methyl as well as benomyl (Delp, 1995). Since 1972 resistance has been reported in well over 100 pathogen species, on more than 50 crops, in dozens of countries. The commercially significant spectrum of the B1 MBC comprises most ascomycete fungal pathogens including the problematic BOTRCI groups and the powdery mildews. Essentially all ascomycete species that have been exposed to MBCs have developed field resistance, in all regions where resistance has been studied.

There appears to be no fitness penalty associated with these target site mutations. The resistant mutants quickly take over the population in affected species and cessation of spraying even for several years has not resulted in the reappearance of the susceptible wild types.

No other mechanism of resistance has been found for the MBC fungicides. This is consistent with the genetically dominant MOR. Production of excess amounts of β-tubulin through gene overexpression or duplication has a severe effect on nuclear division and prevents mycelial growth, conferring a severe fitness penalty. This is because β-tubulin operates as a protein complex with several other proteins to form microtubules. An imbalance in the supply of microtubule proteins disrupts their formation. Also, no cases of efflux pump resistance have been found. It may be that the common form of target site mutation is so effective, with the high RF and an undetectable fitness penalty, that they evolve before efflux mutants would appear.

The discovery of MBC resistance led to screens for leads that were active against the mutant strains. This led to the discovery of the *N*-phenylcarbamate fungicide, diethofencarb. Diethofenocarb was found to efficiently control E198A strains of PYRPBR, COLLGL, BOTRCI, MONILA and VENTIN. However, the E198K and F200Y strains were insensitive to both B1 and B2 fungicides. A similar pattern of negative cross-resistance was seen with the B3 new benzamide fungicides zoxamide and ethaboxam. B3 fungicides have field activity against oomycetes but in the laboratory, the ascomycete strains carrying E198A but not E198K or F200Y isolates that are resistant to benomyl are sensitive to diethofencarb and zoxamide (Leroux, 1992; Malandrakis *et al.*, 2011).

The discovery of negative cross-resistance between B1 and B2 fungicides and the commonest β-tubulin mutation E198A stimulated the design of schemes to exploit the observation to generate infinitely sustainable fungicide sensitivity. Schemes were put forward whereby the two fungicides were alternated and where they were used in mixtures. In the former case, MBC fungicides would be used until most of the population carried the E198A mutation. Then the disease would be treated with diethofenocarb until the population returned to the E198 wild-type genotype. Using a mixture might be expected to prevent at least the commonest mutations from appearing. Neither scheme, nor any other exploitation of negative cross-resistance, has been used outside experimental plots. A combination of cost, fears that different mutations would appear, and regulatory difficulties has combined to consign this idea to the 'too hard' basket.

MBC fungicides have been withdrawn from many countries and markets. The original developer of benomyl, DuPont, ceased manufacture in 2001. None the less, fuberidazole, thiabendazole and thiophanate-methyl are still used in the EU, Australia and elsewhere around the world. FAO data suggest that 3400 t of MBC fungicides were used in 2017 with Argentina, Myanmar, Japan and Poland being the major users (FAOSTAT).

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C3; Quinone outside inhibitors

Resistance to QoI fungicides appeared within 2 years of their introduction around 1996. Resistance has emerged in about 40 pathogen species and many countries within a maximum of 12 years of introduction. A few species have so far escaped resistance despite conditions that would be expected to select for such mutants. These species include PHYTIN and RHYNSE (Bartlett *et al.*, 2002; Gisi *et al.*, 2002). Major examples are SEPTTR, UNCINE and other powdery mildews. RF values are often very high (>100) and while all curative activity is lost, some preventive activity remains for some fungicides in this class.

The target site of QoIs is cytochrome b. The gene encoding this protein is found in the mitochondrial genome, which led some theorists to predict that it would be protected from resistance. Many mitochondria are found in each cell and each organelle contains multiple copies of the circular DNA genome. It was therefore expected that resistant mutations would be unlikely to evolve. Instead, and to the considerable chagrin of all involved, a very consistent pattern emerged whereby a single mutation designated G143A was found in this gene in the great majority of the affected pathogens. In a few cases, the F129L mutation has been found but this is associated with lower RFs especially for pyraclostrobin (Table 11.6). Two recent cases of resistance associated with a third site, G137R/S, have been reported. One of these species is the oomycete PHYTCP. This is significant as hitherto no resistance had been found in the *Oomycota*.

There was complete cross-resistance with G143A and all QoI actives but no other fungicide classes. There also appears to be no significant fitness penalty associated with resistance. Mutations in this region of the protein prevent docking of the fungicide and fully explain the resistance (Gisi *et al.*, 2002). It is interesting that the fungus that produces the lead compound, *Strobilurus tenacellus*, has a cytochrome b protein with different amino acid at the G143 position.

The identification of the MOR as a change in the sequence of the *Cytb* gene led researchers to develop PCR assays to monitor populations. The *Cytb* gene is highly conserved and so primers can be designed that amplify the region from different species. Comparison of this region in the wheat tan spot pathogen PYRNTR and the barley net blotch pathogen PYRNTE identified that the latter had an intron which interrupted the codon for the glycine at position 143 (Sierotzki *et al.*, 2007). Both pathogens had isolates with moderate RF with the F129L mutation; this is of little field significance especially when actives such as pyracolstrobin are used. However only tan spot had the G143A mutation, and these had large RFs and were uncontrollable in the field. An intron in the 143 codon of PYRNTE prevents the selection of the G143A mutation. The nucleotide change needed to change the codon from G to A alters the splice site such that the pre-mRNA cannot be successfully processed. Such mutations are lethal. This led researchers to quickly scan other target genomes for what has become known as the 'blessed intron'. Introns have been found in rust mitochondrial genomes, thereby explaining their failure to develop G143A resistance to QoIs. Recently PHAKPA has developed F129L resistance confirming that rusts have no inherent protection against developing fungicide resistance (Klosowski *et al.*, 2016). This was also the case in BOTRCI (Yin *et al.*, 2012). The presence and number of introns in various species vary markedly and do not follow the phylogeny of the species. Therefore, it is by no means impossible that intron-free isolates of species exist somewhere in the world. As such isolates would be vulnerable to G143A resistance,

Table 11.6. *Cytb* mutation position and changes – archetype SEPTTR. (Authors' own table.)

Position and change	Species
F129L	SEPTTR, ALTETO, PHAKPA, PLASVI, PYRIOR, PYRNTE, PYRNTR, RHIZSO
G137R/S	PHYTCP, CLADCA
G143A	ALTEAL, ALTESO, ALTELY, ALTETO, BOTRCI, CERCBE, CERCBE, COLLGR, ERYSGH, ERYSGT, LEPTNO, MICDMA, MONGNI, MYCOFI, PLASVI, PLEOAL, PODOFU, PSPECU, PYRIOR, PYRNTR, RAMUCC, RHIZSO, RHYNSE, SEPTTR, UNCINE, VENTIN

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we should therefore remain vigilant for resistance even for species where the examined populations contain these introns.

The early and dramatic appearance of resistance to QoIs in so many very important pathogens galvanized the industry into developing resistance management tools. The most important was to use QoIs only in combination with another fungicide, normally a triazole or SDHI or a multi-site. Azoxystrobin is sold as a mixture with cyproconazole in the product Amistar Xtra; pyraclostrobin is sold as a mix with epoxiconazole in Opera. This improves the spectrum and modelling studies indicate it will lengthen the effective life of the products (Hobbelen *et al.*, 2011). In addition to mixtures, alternations of fungicides are also recommended. As a result of these actions, sales of QoIs have remained very strong. With their very low mammalian toxicity, the QoIs have a secure place in the market for many years to come.

C2; Succinate dehydrogenase inhibitors

The rapidly growing family of SDHI fungicides has become a mainstay of crop protection since 2006 particularly since resistance hit the efficacy of QoIs and DMIs. The first SDHIs (from 1966) were carboxin and oxycarboxin but their spectrum was limited to seed-borne and soil basidiomycetes such as the smuts and bunts. Resistance was observed but was of minor practical importance. Since 2006, a series of new compounds has been introduced with broad-spectrum activity against fungi and they have rapidly increased their market share.

Succinate dehydrogenase (SDH) is made up of four proteins, SDH-A to -D, each encoded by an unlinked nuclear gene and synthesized in the cytosol, imported into the mitochondrion, and embedded in the inner mitochondrial membrane along with the other complexes of the electron transport chain. SDHIs act by binding to and inhibiting the complex, leading to reduced electron flow and oxidative damage. Subunits B, C and D form the binding pocket for ubiquinone. The genes for the SDH subunits are not perfectly conserved between affected species. Therefore, the homologous amino acids have different numbers in different species, making comparisons cumbersome. The barley net blotch pathogen PYRNTE is the species with the most described mutations and so it was chosen as the archetype species (Mair *et al.*, 2016). Alignments of the affected species' genes with the PYRNTE genes identify amino acids where mutations associated with resistance are commonly found. The numbering used here refers to the position of the amino acid in the PYRNTE genes and is referred to as the mutation label. To avoid confusion, it is also necessary to specify the subunit. For example, mutations to resistance have been found in both SDH-C H134R and SDH-D H134R, distinguished as C-H134R and D-H134R (Tables 11.7, 11.8 and 11.9). Homology-based structures of SDH complexes have been determined and have identified a number of residues that interact with the ubiquinone and the Fe/S clusters involved in electron transport (Ishii and Hollomon, 2015; Stammler *et al.*, 2015). These include SDH-B P230, referred to as B-P230, plus B-H277, B-T278, C-T68, C-W69 and D-D129. Italics are used to describe amino acid sequences in other species such as USTIMA.

Early research found a resistant strain of USTIMA that harboured the *B-H277L* mutation (Broomfield and Hargreaves, 1992). Later, laboratory mutants of SEPTTR were found with two different mutations at the same site, *B-H277Y,L* (Skinner *et al.*, 1998). Transformation of this tractable species with the *B-277Y* version showed conclusively that this mutation conferred the resistance and identified the target site. These studies demonstrated that single mutations could generate strains with high RFs in different species.

Table 11.7. SDH-B mutation position and changes – archetype PYRNTE. (Authors' own table.)

Position and change	Species
P230L/T/F/H	SEPTTR, BOTRCI, PLEOAL
N235T/I	PYRNTE, RAMUCC
H277Y/R/L/N	PYRNTE, UNCINE, EUROOR, USTIMA, SEPTTR, SCLESC, BOTRCI, BOTREL, PLEOAL, ALTEAL, ALTESO, DIDYBR, CORYCA, PODOXA, USTIMA
T278I/A	VENTIN, SEPTTR

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Table 11.8. SDH-C mutation position and changes – archetype PYRNTE. (Authors' own table.)

Position and change	Species
S/A73P/V	CORCYA, SEPTTR, BOTRCI
N75A/S/K	PYRNTE, SEPTTR, RAMUCC
G79R	PYRNTE, SEPTTR, RAMUCC
H134R	PYRNTE, ALTEAL, SCLESC, RAMUCC, ALTESO, RHIZCE
S134R	PYRNTE, ALTEAL
R140M/S/T	SEPTTR
H141R	SEPTTR, VENTIN, RAMUCC
G159D/S	UNCINE, RAMUCC

Table 11.9. SDH-D mutation position and changes – archetype PYRNTE. (Authors' own table.)

Position and change	Species
D124E/N	PYRNTE, ALTEAL, ALTESO
H134R	PYRNTE, BOTRCI, SCLESC, ALTEAL, ALTESO
D145G/E	PYRNTE, EUROOR, SEPTTR

Since 2006, a range of other SDHI fungicides has been released and resistant mutants have been found in many species within 3–6 years. The mechanisms of resistance have so far been almost entirely restricted to point mutations in the SDH-B, -C and -D subunits, and the residues mentioned above plus several others have been implicated. The absence so far of resistance due to gene overexpression may be related to the tetrameric nature of the complex. We can predict that coordinated overexpression of all the subunits would be needed to overcome a competitive inhibitor.

The RFs associated with these mutations vary from 1 to >100 depending on the species, mutation and inhibitor. One study used site-directed mutagenesis in BOTRCI to make many isogenic strains mutated at SDH-B *P230F/L/T*, *N235I* and *H277L/R/Y* (Lalève *et al*., 2014b). These strains were tested against boscalid, fluopyram and carboxin for EC_{50}s and for enzyme activities. There were good correlations in EC_{50}s between these isogenic strains and field strains with the same mutations indicating that SDH mutations account well for the observed resistance. The SDH activity of the mutants was substantially reduced in all cases except *H277L*. This suggests that a significant fitness penalty would apply to most of the mutations. Measurements of fitness penalties have been carried out in several studies with mixed results (Kim and Xiao, 2011; Fraaije *et al*., 2012; Lalève *et al*., 2014a; Veloukas *et al*., 2014). Fitness penalties are observed more frequently in BOTRCI than in other species. This may reflect the rapid life cycle and prolific sporulation of BOTRCI making penalties easier to detect.

The new generation of SDHI fungicides was classified by FRAC as being medium to high risk due to their single site of action, high RFs and modest (at best) fitness penalties. These predictions have been borne out and resistance has been detected in ten or more pathogen species. Different SDHI fungicides appear to be selecting different mutations. Boscalid is associated with the B-*H277Y/R/L/V* mutations whereas the pyrazole SDHIs such as bixafen are associated with the *C-H134R* mutation. Cross-resistance between the different SDHIs is generally high, but the pyridinylethyl benzamide fluopyram shows low correlations and even negative cross-resistance with other SDHIs.

Not all cases of evolved SDHI resistance can be ascribed to a mutation in one of the SDH subunit genes. Strains of SEPTTR with resistance to fluopyram and isofetamid, but not other SDHIs, have been characterized and these strains do not carry mutations in SDH-B, -C or -D associated with resistance. Non-target site resistance is therefore postulated but still needs to be confirmed and characterized (Yamashita and Fraaije, 2017).

Recently a new mechanism of resistance to SDHIs has been discovered in SEPTTR (Steinhauer

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et al., 2019). Whereas most species have one gene for each of the four subunits, SEPTTR was found to have a second gene for SDH-C called *ZtSDHC3*. The alternative SDH-C protein forms a fraction of the active SDH complexes. Such complexes are relatively resistant to a subset of SDHIs which include fluopyram. Paralogue SDH subunits were found in other *Capnodiales* such as RAMUCC and may explain the rapid development of resistance in these species.

Resistance to SDHIs is spreading quickly and seems to follow introduction and intensive use of these fungicides with a gap of about 3 years. When the new SDHIs were introduced, the lessons from the history of resistance to MBC and QoI fungicides were applied and in almost all cases, SDHIs are sold as mixtures, mainly with multi-site fungicides.

G3; Demethylation inhibitors

Resistance to G1 DMIs crept up slowly over a period of 30 years and was not recognized as a serious issue until the mid-2000s. DMIs were the mainstays for disease control especially in cereals since the 1970s. Unlike the MBCs and QoIs, there were no obvious cases of catastrophic failure to catch the attention of the industry. Indeed, for a while it was believed that DMIs were immune to resistance unlike the MBCs and QoIs. However, mounting evidence accumulated that there was a gradual decline in the observed efficacy of certain DMIs ascribed to various factors.

Research into resistance to medical DMIs and laboratory studies prepared the ground (Hippe and Koller, 1986), but it was not until 2001 that DMI resistance was linked to genetic changes in field isolates of plant pathogens (Schnabel and Jones, 2001). Since then, a plethora of studies have been published which detail the pattern of cross-resistance, RFs and MORs (Cools *et al.*, 2013).

Growers were reporting that they were having to use higher and higher doses to achieve the same level of control. When strains from these fields were examined, the RFs were found to be moderate: 20–50. This explains why catastrophic failures were never found. Furthermore, whereas some older DMIs were obviously suffering from resistance, newer DMI fungicides remained as potent as upon release. The steady supply of new DMI actives served to camouflage the steady evolution of resistance over a period of 40 years.

The research has highlighted three MORs:

1. Target site alteration leading to reduced sensitivity to some DMIs.
2. Target site overexpression enabling the fungus to survive higher doses of fungicide.
3. Non-target site mutations in efflux pump genes.

The target site for the DMI fungicides is the CYP51 C14-demethylase. In many fungi this is encoded by a single gene called *Cyp51B*. Other fungi have a second CYP51 encoded by *Cyp51A* and a few have a third encoded by *Cyp51C*. Some species have two or three *Cyp51A* and *B* genes. Overexpression of one paralogue appears to confer resistance. Some cases of overexpression are due to gene duplication.

The archetype sequence for CYP51A is the ASPEFU sequence and for CYP51B is the SEPTTR sequence (Mair *et al.*, 2016). Mutations in *Cyp51A* affect mainly fungi of clinical rather than agricultural relevance but now include PYRNTE. No mutations in the target site of CYP51C have yet been found.

Mutations in the coding region have been found at seven sites with at least two changes and/or in at least two species (Tables 11.10 and 11.11). These sites must be relevant amino acid changes. A further 22 sites in *Cyp51A* and 39 sites in *Cyp51B* have mutations found only in one case. There is often solid evidence that these sites are also relevant from modelling or functional studies.

The 53 affected sites often appear in combinations; in SEPTTR 30 variant sites have been found in 70 combinations (Cools *et al.*, 2013; Hawkins and Fraaije, 2016). Analysis of the impact of each of these versions on fungicide activity of the 40+ DMI fungicides is a substantial

Table 11.10. CYP51A mutation position and changes – archetype ASPEFU. (Authors' own table.)

Position and change	Species
G54E/K/R/V/W	ASPEFU, ASPEPA
Y121F	ASPEFU
M220K/I/T/V	ASPEFU
D280	ASPEFU, ASPEFL
M286	ASPEFU, ASPEFL
F495I/L	ASPEFU, PYRNTE

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Table 11.11. CYP51B mutation position and changes – archetype SEPTTR. (Authors' own table.)

Position and change	Species
D/E107V/K	SEPTTR, PSDCHE
V136A/C/G	SEPTTR
Y137F/H	SEPTTR, CANDAL, PHAKPA, PUCCRT, ERYSGH, ERYSGT, MONIFC, UNCINE, PENIDI, FILBNF, GIBBZE, USTNVI, LEPTNO
K148R/E/Q	PHAKPA, CANDAL, ERYSGH
A311G	SEPTTR, MYCOFI
A379G	SEPTTR, MYCOFI
L385S/L	CANDAL, COLLDU
A/S410T/F	SEPTTR, CANDAL
Y459C/D/N/S/P/Δ	SEPTTR, MYCOFI
G460D/Δ/A	SEPTTR, MYCOFI
Y461D/H/S/N	SEPTTR, CANDAL, MYCOFI
G476S	SEPTTR, PENIDI, PYRPBR, CANDAL
I483T	CANDAL, PHAKPA
S521Q	PSDCHA, PSDCHE
S524T	SEPTTR, PYRPBR, ERYSGH

undertaking that is technically much simpler when the relevant gene is expressed in the yeast SACCCE (Cools *et al.*, 2010). The results from yeast and the original fungi show that single mutations generally have a small impact on RFs corresponding to little effect on agronomic performance. However, multiple mutations, up to seven in SEPTTR, can give substantial RFs (>100) that correspond to field failure.

Target site mutations affect different DMI actives in different ways. Cross-resistances are mostly positive, but the correlations are generally weak. A few cases of clear negative cross-resistance have been observed (Leroux *et al.*, 2007) particularly between fungicides from the different chemical groups, triazoles (including prothioconazole), imidazoles (especially prochloraz) and pyrimidines (especially fenarimol).

The current situation has been likened to a 'rugged adaptive landscape' (Hawkins and Fraaije, 2016), adopting a convention used in evolutionary biology. The application of a selection pressure selects individuals that are fitter in the prevailing conditions; such fitness is said to place them 'higher in the landscape'. If the landscapes were smooth, populations could evolve to gradually climb the landscape and thereby evolve ever greater fitness. A single genotype corresponding to the fittest phenotype would be consistently selected. However, if the landscape is rugged, with many peaks divided by deep troughs, pathogens are unable to traverse a valley to climb a distant, higher peak and can get stuck at local peaks.

A given fungicide selects sequentially for one or more mutations in the *Cyp51* gene(s). Each mutation provides an advantage in the landscape. It is important to recognize that a mutation with even a very small RF will be selected by a fungicide even if the agronomic impact is small if the fitness penalty is also small. Several mutations accumulate one after the other to allow the fungus to climb the fitness slope. But the landscape provided by modern agricultural practices with DMI fungicides is not smooth. Different fungicides select for different mutations and the first mutation selected by one fungicide may be deleterious for survival in the presence of another.

The first fungicides used in many markets were flutriafol, triadimenol, tebuconazole and propiconazole. These fungicides selected the *Y137F* mutation. Continued selection with the early triazoles leads to double mutations with *S524T* and substantial loss of control as seen with ERYSGH in Australia (Tucker *et al.*, 2020). A similar picture emerges from studies of SEPTTR from Oregon. Isolates collected before and after the use of triazole fungicides were compared (Estep *et al.*, 2015). The main fungicides used were propiconazole and tebuconazole. The genotypes collected after fungicide use started were dominated by the mutations *G460D* and *Y137F*.

In primary markets, these early DMIs were gradually replaced with second- and third-generation DMIs not primarily because of resistance (although resistance may have been occurring

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undetected), but for better spectrum and activity. The later DMIs are associated with different patterns of resistance. The mutations at *456/460/461* are associated particularly with epoxiconazole and the *A379G* with prothioconazole.

The highly complex pattern of target site mutations and DMI resistance is also highly dynamic. Predicting which fungicides will work best is a multidimensional problem that needs to take account of the density of the pathogen, the frequency of each of up to 70 different genotypes, any fitness penalties and the EC_{50}s for each of the genotypes to the 40 or more DMI fungicides that can be used, solo or in mixtures. The very complex genotypes found in SEPTTR are individually rare. This implies that each genotype is advantageous in one field situation but deleterious compared with other genotypes in a neighbouring field in which a different fungicide regime is applied. An increase in the efficacy of prochloraz has been observed in recent years and this is likely due to the negative cross-resistance seen between mutations favoured by popular fungicides such as prothioconazole and epoxiconazole and those selected by prochloraz.

Target site resistance to DMIs in SEPTTR is found in Europe, North America, North Africa, North America and, more recently, New Zealand and Australia (Boukef *et al.*, 2012). Australian isolates were from Tasmania and Victoria and the *Cyp51* genes exhibited up to five mutations in the same strain (McDonald *et al.*, 2019).

Overexpression of *Cyp51A* and *B* genes has also been linked to resistance (Schnabel and Jones, 2001; Cools and Fraaije, 2013; Hawkins *et al.*, 2014; Omrane *et al.*, 2017; Mair *et al.*, 2019). This phenotype is linked to insertions in the promoter of the gene. The RFs are in the range of 7–15 and are the same regardless of which DMI is tested. The interpretation is that the CYP51 enzyme is working at near full capacity during fungal growth. Inhibition by a DMI therefore has a noticeable effect on flux through the pathway and this can be detected as both a reduction in growth rate and the accumulation of toxic sterols (Bean *et al.*, 2009). Overexpression of the gene produces more enzyme and therefore compensates for the reduction in specific activity. The insertions in the promoter have been found in several species.

Although definitive studies are lacking, theoretical considerations would suggest that the cross-resistance to all DMIs would be strongly positively correlated when it is the *Cyp51B* gene that is overexpressed. When the *Cyp51A* gene is overexpressed, the impact on different DMIs would depend on the sensitivity of the overexpressed gene product. In VENTIN overexpression of *Cyp51A* led to difenoconazole resistance but not myclobutanil (Villani *et al.*, 2016). In RHYNSE (Hawkins *et al.*, 2014) overexpression of *Cyp51A* was partially linked to tebuconazole and propiconazole resistance.

Overexpression is linked in many cases to an insertion element in the promoter of the *Cyp51* gene in SEPTTR, PHAKPA and PYRPBR (Ghosoph *et al.*, 2007; Cools *et al.*, 2012; Carter *et al.*, 2014; Schmitz *et al.*, 2014). This means that methods to genotypically screen for resistance are technically facile.

H5; Cellulose synthase

Three chemical classes of fungicide target cellulose synthase and thereby control only the oomycete pathogens. Resistance has been linked to mutations in the target gene *CesA3* of PHYTIN, PLASVI and PSPECU (Blum *et al.*, 2010a,b, 2012) and were observed as quickly as 2 years after introductions. Two sites have shown point mutations in three species, albeit only in laboratory mutants of PHYTIN and PHYTCP. The insensitivity of *Pythium* species is linked to their possession of L or M at position 1109, equivalent to the mutant genotypes of naturally sensitive *Phytophthora* and downy mildew species (Table 11.12).

A1; Phenylamides

The PA fungicides metalaxyl, benalaxyl, metalaxyl-M, benalaxyl-M and furalaxyl remain important market leaders in the control of oomycete diseases. Resistance to these fungicides was observed within 2–3 years of their introduction in European markets and is currently common

Table 11.12. *CesA3* mutation position and changes – archetype PHYTIN. (Authors' own table.)

Position and change	Species
G1105A/V/S/W	PHYTIN, PLASVI, PSPECU
V1109L/M	PHYTIN, PHYTCP, PHYTDR

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in many of the oomycete pathogens and in many parts of the world.

The MOA has long been associated with inhibition of RNA polymerase, but efforts to link resistance to particular genes have remained frustrating. A continuous spread of RFs from 10 to 1000 has been observed (Müller and Gisi, 2012; Tian *et al.*, 2016). A strong association between a mutation in an RNA polymerase gene (RNAPol1; RPA190) denoted Y382F and resistance was noted in a cross between sensitive and insensitive PHYTIN (Randall *et al.*, 2014). However, this is not the only mechanism of resistance (Childers *et al.*, 2015; Matson *et al.*, 2015; Montes *et al.*, 2016). Resistant populations were genetically distinct from sensitive populations and the resistance is sometimes unstable, suggesting a biochemical rather than genetic mechanism. Evidence that resistance incurs a fitness penalty has been obtained (Wang and Ma, 2015) and this may account for the continued efficacy of PA fungicides, despite the high risk of resistance. Universal methods to screen for resistance genotypically are not yet available and hence studies of resistance in field isolates remain laborious.

Multi-drug resistance

Most cases of fungicide resistance affect only one fungicide class and are due to alteration in either the structure or amount of the target site gene product. In the last decade or so, isolates of fungicide with resistance to many different classes of fungicide have emerged and are now regarded as a significant and major threat to agricultural crop protection. MDR is characterized by resistance to many if not all classes of fungicides. It may be no coincidence that MDR phenotypes have become more common since mixtures and alternations of fungicides suffering from single-site resistance were used. MDR therefore calls into question resistance management strategies that rely on fungicide mixtures and alternations that are likely to select for MDR phenotypes.

MDR phenotypes are due to enhanced activity of membrane proteins that function to pump fungicides from inside the fungal cell into the external medium. They therefore reduce the concentration of the fungicide so that the inhibitory effect is reduced. The natural role of fungal efflux pumps is to export toxic compounds used to combat other microorganisms.

There are two classes of efflux pump found in fungi: the ATP-binding cassette class (ABC) and major facilitator superfamily (MFS) (Hahn and Leroch, 2015). ABC transporters are encoded by up to 50 genes and are expressed at low levels during unstressed growth. The pumping action needs ATP. In contrast, MFS proteins' export of fungicides is coupled with the import of protons. MDR resistance is normally caused by overexpression of genes encoding ABC or MFS linked to insertions of short regions of DNA into their promoters. The ready availability of fungal genomes has facilitated the genetic dissection. Molecular assays can often be developed that detect known insertion elements.

MDR affects all main classes of fungicides and has been reported in more than 12 pathogens including BOTRCI (Hahn, 2014), PYRNTR (Reimann and Deising, 2005), FUSASO (Kalamarakis *et al.*, 1991), PENIDI (Sánchez-Torres and Tuset, 2011), PSDCHE (Leroux *et al.*, 2013), SCLEHO (Hulvey *et al.*, 2012) and MONIFC (Luo and Schnabel, 2008). MDR phenotypes are often due to the upregulation of pump genes and so genotypic monitoring is complex. However, a 519 bp insertion in the *MgMFS1* gene of SEPTTR was found in 50% of the MDR strains (Omrane *et al.*, 2015). MDR phenotypes are most frequently reported in BOTRCI and linked to both MFS and ABC transporter mutations. Resistance is found at frequencies of up to 50%. RFs of 5 to 15 are commonly reported.

The fitness of the MDR strains of pathogenic fungi is widely assumed to be compromised because the transporters need direct inputs of ATP or protons to drive drug efflux. However, the steady increase in the number of reports and frequency of MDR isolates would suggest the fitness penalty is not a significant factor limiting the prevalence of MDR isolates under current conditions. The presence of MDR at even low levels suggests that the use of multiple fungicides, whether in mixtures or alternations, is selecting for MDR strains and calls into question this plank of resistance management.

The Management of Resistance

Fungicide resistance is now recognized as a fact of life for the fungicide industry. Therefore, a

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series of practices has been recommended by fungicide manufacturers and national agricultural advisory services. A typical example is the advice collated by the UK-based Fungicide Resistance Action Group (FRAG). Its advice is based on the premise that 'Good resistance management is based on limiting the level of exposure of the target pathogen to the fungicide'. Hence FRAG advises the following nine concepts:

1. Fungicide input is only one aspect of crop management and other control measures should always be used, such as good hygiene through disposal of crop debris and control of volunteer crops which may harbour disease.
2. Always aim to select varieties exhibiting a high degree of resistance to diseases known to be prevalent in your area, in addition to the main agronomic factors you desire.
3. Avoid growing large areas of any one variety, particularly in areas of high disease risk where the variety is known to be susceptible.
4. Only use fungicides in situations where the risk or presence of disease warrants treatment.
5. Use a dose that will give effective disease control, and which is appropriate for the cultivar and disease pressure.
6. Make full use of effective fungicides with different MOAs in mixtures or as alternate sprays.
7. Ensure that mixing partners are used at doses that give similar efficacy and persistence.
8. Monitor crops regularly for disease and treat before the infection becomes well established.
9. Avoid repeated applications of the same product or MOA and never exceed the maximum recommended number of applications.

Some of these pieces of advice have been validated by experiment or by modelling whereas others are considered to be self-evident. The premise 'Good resistance management is based on limiting the level of exposure of the target pathogen to the fungicide' recognizes the truism that selection for fungicide resistance can only ever occur when the pathogen is exposed to the fungicide. Herein lies the conundrum. A farmer will only use a fungicide if it gives useful control, and this inevitably exposes the pathogen to the fungicide. The goal is to achieve satisfactory disease control while delaying or preventing the development of resistance.

Good agronomy hygiene

Several of the pieces of advice aim to reduce the total amount of the pathogen in the environment of the crop. Thus advice statement #1 recommends destroying volunteer crops and infected crop debris and using clean seeds. The retention of crop debris is clearly associated with several important diseases (Jørgensen and Olsen, 2007). However, limited-tillage techniques are critical for the success of farming in most of the drier arable zones around the world.

Proper agronomy and irrigation are fundamentals of IPM and are equally essential: excessive nitrogen fertilization promotes the development of foliar disease in multiple crops; sowing or planting date can avoid some diseases while discouraging others; crop density can suppress or exacerbate disease, depending upon crop or pathogen (Jørgensen *et al.*, 2014).

Integrated disease management

Advice statements #2 and #3 acknowledge that genetic disease resistance is a critical part of disease management even when a pathogen is well controlled by the fungicide. Plant breeders combine a multitude of traits in order to generate successful cultivars. Disease resistance is only one of these traits and by no means the highest priority in most cases. It is rare therefore for a crop variety to be adequately resistant to *all* the pathogens likely to infect it. A farmer may feel obliged to use a fungicide if even only one disease threatens the crop. And as most fungicides are broad-spectrum, it may be considered that the genetic disease resistance is superfluous.

A further conflict can arise if a crop variety that is resistant to the pathogens of importance has a lower yield than one that is susceptible in the absence of disease. This is known as a 'yield trade-off' (Brown and Rant, 2013). A farmer may calculate that a $20 fungicide spray on a susceptible cultivar may be more profitable than using a cultivar that is resistant but gives a 200 kg lower yield.

The advice on growing a single resistant variety is based on the risk that the pathogen may evolve virulence and thus create an epidemic. This advice underpins the concept of integrated

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disease (or pest) management. IDM (or IPM) embodies the advice that all control methods should be applied. In this way, the fungicide protects the genetic disease resistance because any strain that evolves virulence would be controlled by the fungicide; vice versa, any strain that evolved fungicide resistance would be controlled by the genetic disease resistance.

IDM emphasizes the value of diversity in all its aspects. Diversity in fungicide is covered by advice statement #6. Another aspect of IDM is the use of crop rotations so that pathogen residues are kept to a minimum from season to season. Yet another is to use mixtures of cultivars in the same field. The concept is that if two cultivars have different disease resistance profiles, pathogens will spread more slowly on the mixture that they would in pure cultivar stands. A recent meta-analysis of wheat SEPTTR trials supports the idea, showing significant disease reductions and yield increases especially when fields were not treated with fungicide (Kristoffersen *et al.*, 2020). Cultivar mixtures would not suit every scenario. The cultivars must mature at very similar rates so that they can be harvested at the same time. Also the grains must fulfil the same market niche. But with so many fungicides being removed from the market and rendered ineffective by resistance, all options need to be considered.

Dose rate

Advice statements #3 and #4 can be summarized as using the minimum quantity of fungicide that gives adequate disease control. In the absence of disease, there is clearly no need to use any fungicide. To some extent, this conflicts with advice statement #8 to spray before the disease gets established. In practice, most growers will know from experience which diseases are likely to occur and which weather patterns promote their spread. In these cases, spraying early is prudent and conforms with the overall premise of 'limiting the level of exposure of the target pathogen to the fungicide'. Spraying early reduces the total number of pathogen spores that get exposed to the fungicide and hence the chance that a resistant mutant will be subjected to the selection pressure.

The effect of dose on the emergence of resistance was for a long period the subject of intense debate but it is now established in a great variety of cases that the lower the dose the lower the risk of resistance. This result is supported by both modelling and experience and has become embodied in official advice and farmer practice in many countries (van den Bosch *et al.*, 2011, 2014a,b; Jørgensen *et al.*, 2017; Mikaberidze *et al.*, 2017). Rationalization of this finding stems from the simple idea that the resistant isolates of the pathogen survive with higher frequency at all non-zero doses of the fungicide (Fig. 11.6). The selection pressure is represented by the vertical arrows and is higher at higher doses. Figure 11.6a models a fungicide resistance with a moderate RF. Figure 11.6b represents a high RF; the selection pressure still increases with increasing dose. Figure 11.6c represents a fungicide resistance with a significant fitness penalty. Here the selection pressure is negative at low doses and increases with dose.

The concept that low dose equates to low risk was counterintuitive for many and contrary to the established advice for herbicide and insecticide resistance. For the fungicide companies it meant selling less fungicide in the current year but with the promise that sales will continue for a longer period. With weeds, a high dose can eradicate a weed population and therefore a grower can be sure that no resistant mutant has survived. If a weed survives a herbicide spray, it can be detected and killed by another herbicide, by mowing, grazing or even burning. Pathogen populations are huge and invisible and so no warning of resistance occurs.

The effect of ploidy is also much discussed. Weeds are diploid or polyploid and most herbicide resistance traits are semi-dominant. So, if one allele of a herbicide tolerance gene mutates, this heterozygous plant would survive a moderate dose, higher than the sensitive homozygote but lower than the resistant homozygote. The chances of both alleles mutating simultaneously are tiny. Hence growers are advised to use a dose of the herbicide that would kill the heterozygous resistance plant. If such plants were allowed to grow some would cross-pollinate, and this would create homozygous mutants that can tolerate much higher doses. Most pathogens are haploid and so the concept of heterozygous resistance does not apply. However, many pathogens are

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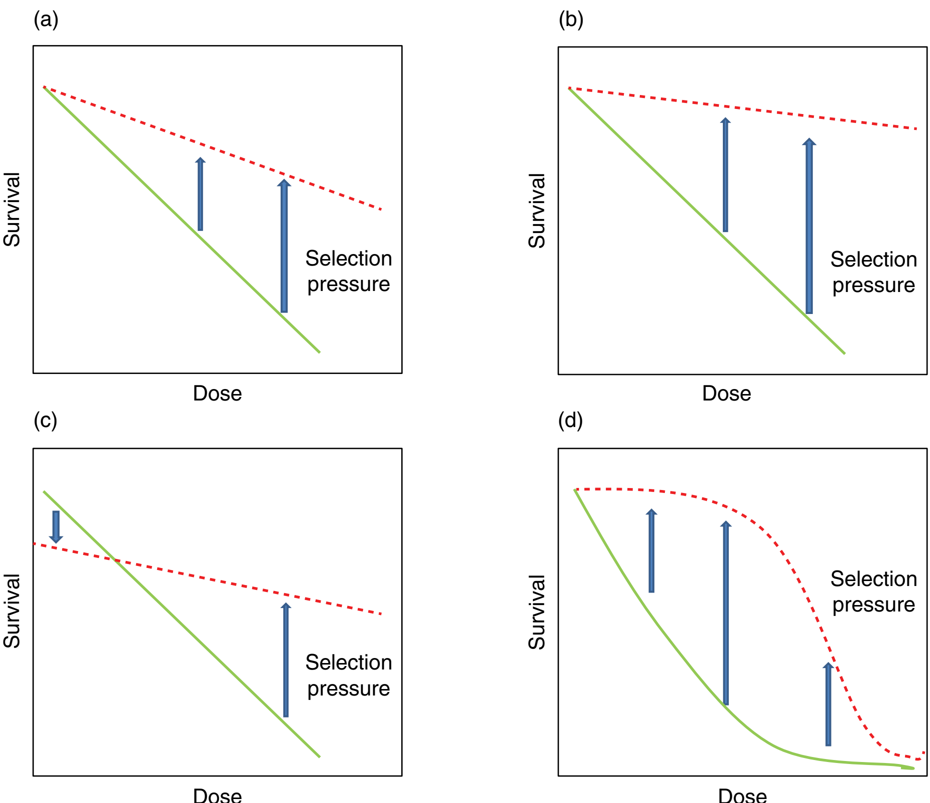

Fig. 11.6. Schematic dose–response curves for wild type (——) and resistant mutant (– – –). Panel (a) represents a mutant with a moderate resistance factor (RF) and shows that the selection pressure (vertical arrow) is higher at higher doses. Panel (b) shows a mutant with a high RF; the selection pressure still increases with increasing dose. Panel (c) represents a mutant with a fitness penalty at low dose; the selection pressure at low dose is therefore negative. Panel (d) represents a scenario in which the survival of the mutant and wild type converge at high dose; in these conditions (so far not observed in fungi although seen in weeds) the selection pressure may decrease at high dose.

either diploid (like PHYTIN) or dikaryotic (such as the rusts) and both have been found to evolve resistance in suitable cases.

A more relevant difference between herbicide resistance in weeds and fungicide resistance in pathogens is that the former is dominated by metabolic resistance mechanisms in which the pesticide is detoxified by enzymes such as the P450 reductases, glucosyl transferases or glutathione *S*-transferases. Low doses of herbicides induce the expression of genes encoding these enzymes, allowing the weeds to survive the dose and set seed. Mutations that increase the speed or degree of induction would therefore be selected by repeated use of doses that killed only some of the weed population. If detoxification mechanism for fungicides become more common, a reassessment of the low dose policy may be needed. Figure 11.6d represents this situation where the survival frequency converges at very high doses. In this case the selection pressure varies both up and down with dose.

Mixtures and alternation

Advice statement #9 argues against the repeated use of the same MOA. Instead statement #6 advises using either mixtures or alternation with different MOAs. Repeated use of the same fungicide MOA applies the selection pressure repeatedly to the already selected population.

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Regulatory authorities therefore legislate for the maximum number of times an MOA can be used in a season.

Mixtures or alternations should be a good way to prevent resistance (Hollomon and Kendall, 1997; Hobbelen *et al*., 2013; van den Bosch *et al*., 2014b). If a strain resistant to one fungicide survived treatment with that fungicide, it would be killed by the other fungicide. For this to be true the MORs need to be different. Hence fungicide companies are increasingly selling fungicides as mixtures of actives with different MOAs. Mixtures of DMIs may provide protection as different DMIs seem to select different mutations (Cools *et al*., 2012, 2013; Cools and Fraaije, 2013).

Modelling studies have supported the notion that mixtures provide several years of protection against the emergence of resistance (Hobbelen *et al*., 2011). In that study, mixtures of high risk (a QoI) and low risk (chlorothalonil) were found to be effective in delaying resistance. The dose of the two fungicides was optimal when the low-risk fungicide was used at the maximum rate and the high-risk one was used at the minimum dose compatible with adequate disease control. This finding equates with advice statement #7 requiring 'that mixing partners are used at doses that give similar efficacy and persistence'. It is self-evident that a fungicide can only contribute to resistance management if it is being used at a dose that would have a significant effect on disease if used on its own. Hence it is necessary for researchers to monitor populations of pathogens for loss of sensitivity to solo fungicides even if that fungicide is only used in a mixture in commercial products. Detection of resistance to one mixing partner would remove the rationale for the mixture.

Mixtures are relatively easy for the farmer as the product is normally sold as such. Farmers can also 'tank-mix' fungicides and add in other pesticides, if appropriate, but some products are incompatible. Alternations of fungicides require extra work on the farm. Theoretical studies suggest that both strategies decrease the risk of resistance for rather similar time periods. Current resistance management practices are based on limitations on dose and on the numbers of times a given MOA can be used in a season together with the use of mixtures and alternations of fungicides from different MOA groups. These resistance management guidelines have been largely validated by practical experience and theoretical studies (van den Bosch *et al*., 2014a).

Multi-drug resistance and resistance management – fungicide refugia?

The use of mixtures of fungicides has become standard practice in the last decade or so. As we have seen, fungal pathogens are adept at evolving resistance to whatever methods of control we put in place so we should not have been surprised that MDR resistance would occur. With MDR, resistance to most if not all MOAs is positively correlated. This would suggest that the only clear way to combat MDR is to avoid using any fungicide over a substantial period and a wide area. This 'refugia strategy' has been used successfully to protect GM insecticide resistance conferred by *Bt* genes (Mallet and Porter, 1992). In the case of insects, the success of refugia is attributed to mating between the susceptible population that thrives on the untreated crop and the resistant population selected by the treated crop. The heterozygotes are controlled if the dose used on the treated crop is sufficient. In the case of fungi where asexual and polycyclic reproduction is the rule, the success of a refuge would need the sensitive population to outgrow the resistant one on the untreated crop. If isolates expressing MDR display even a small fitness penalty, the higher growth rate of the wild-type (non-MDR) strains on a fungicide-free crop would permit the restoration of the population of the MDR-sensitive strain. There are however clear theoretical and practical issues with the use of refugia. The size of the refuge and the susceptibility of the plants grown on it would both need to be big enough to allow the development of a population that could dominate the resistant MDR population within one or at most a few seasons in the local area (Zhan and McDonald, 2013). Furthermore, the deliberate creation of a large pathogen population would run counter to a generalization of resistance management for target site resistance (as well as crop protection generally), which is that overall pathogen population sizes should be minimized.

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New fungicide groups and resistance – can we predict risk of resistance?

The rise of fungicide resistance seems to be an inexorable process against which we are poorly equipped. The use of mixtures and alternations and recommendations to minimize both the number of applications and dose used have been made, but new cases of resistance continue to appear. The clarified theoretical framework charts a course whereby we can be certain that wherever a fungicide is making an effective contribution to pathogen control, it is also selecting for resistance. New fungicide actives will continue to be required if we are to maintain current levels of crop productivity. Can we predict the risk of resistance prior to the release of a new fungicide?

Predictions of risk are currently based on the pre-release selection and testing of resistant mutants. If mutants can be generated, their fitness and the RFs can be determined. High RFs and high fitness are warning factors. However, it is commonly observed that mutants obtained in the laboratory are not always the ones that emerge in the field. The interpretation of this phenomenon is that some resistant mutants have hidden fitness penalties that are only revealed in the field situation. The use of realistic field-simulating microcosms that can be used to test the fungicides over multiple life cycles of the pathogen would have a better chance of uncovering the relevant mutations. The identification of the mutant would allow study of the MOR (and MOA if this was unknown) and permit the development of genotypic monitoring tools.

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12

Legislation and Regulation

Key Points

- Fungicide companies operate within strict and detailed legislative and regulatory frameworks covering the safety and efficacy of products and manufacturing processes. The laws and regulations differ around the world.
- Regulatory regimes are subject to political as well as scientific factors.
- Fungicide users also operate within a strict regulatory framework designed to protect the environment, the farmer and the public and to produce food that is free from damaging residues of pesticides.
- Consumers generally do not appreciate the safety of current fungicides or their importance in maintaining food security.

Introduction

Fungicides can only be sold and used legally if they have sufficiently low levels of toxicity to other organisms including the crop hosts, non-pathogenic fungi, other organisms in the environment and farmers and consumers. National governments have developed laws and regulations to ensure that fungicides are safe. These regulations govern the registration of new actives and formulations. The use of registered products is governed by ongoing rules contained within the 'label' attached to the product.

These legislative requirements of fungicide registration are primary concerns for fungicide companies and represent the major hurdle in bringing a new active to market. The combined cost of registration, environmental testing and toxicology adds up to an average of $180 million per launched product or more than two-thirds of the total cost. The direct costs of preparing and delivering the registration dossiers alone is estimated at $33 million. A great deal of thought and experimentation goes into predicting and testing the properties of lead compounds to minimize the time and effort spent on compounds that are destined to fail to secure registration. It would be bad enough to have to abandon a compound late in development after perhaps $200 million has been spent on its development. But it would be far worse if a compound was released and subsequently found to have some deleterious effect. The loss of reputation and the payment of compensation to damaged parties could threaten the viability of the company.

The purpose of the legislation is to allow benefits to be obtained while incurring the least possible harm to the manufacturer, user, consumer and the environment. For pesticides, this includes a spectrum of activities from the patenting of a candidate product derived from a synthetic or natural source to the examination of its potential short- and long-term effects on

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humans, animals, plants and the environment. More recently, regulations have been introduced that promote practices designed to prevent fungicide resistance and thus prolong the effective life of the compound.

Traditionally, legislative procedures and regulations have differed between countries. The current goal of standardizing pesticide registration regulations across nations ('harmonization') is intended to improve the effectiveness of industry and government resources and lower the costs associated with risk assessment that are eventually financed by the consumer.

Registration Requirements

The legal requirements that define the process of fungicide development and use also apply generally to pesticides. Effective fungicides are difficult to discover and predictably are subject to many rigorous toxicological and environmental tests. By comparison with pharmaceuticals, the action of using a fungicide to control a crop disease is equivalent to the selective and safe treatment of headaches using aspirin dissolved in water and sprayed in low volume from an aircraft over a town in which some of the sufferers are either inside buildings or have not yet arrived on the scene. Fungicides are not usually applied to single, captive plants in the same manner as a pharmaceutical is used on a single patient. Consequently, factors other than safety to an individual become important in determining their safety. An outline of the testing processes and the timescale is given in Fig. 12.1.

Prior to their sale in any country, new and effective products must be shown to be safe to:

- the operator who handles and applies the product;
- the consumer of the treated crop;
- the environment; and
- the crop.

In all countries, the product must be shown to be safe to the operator, to non-target organisms and to the environment. Some, but not all, countries require that the product can be demonstrated to be effective in controlling disease and confer a significant yield increase and/or quality. This extra registration evidence means that field trials must be carried out for each crop and each pathogen in a representative range of

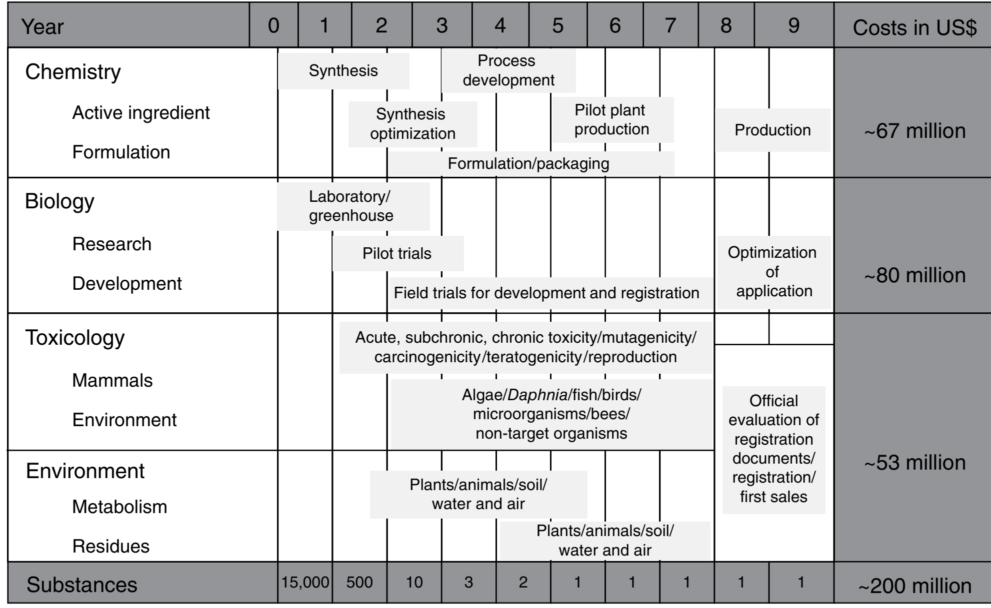

Fig. 12.1. Development of a crop protection product. (From an ECPA study carried out by Phillips McDougall. © Phillips McDougall.)

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agroecological zones. This represents a substantial costs burden when compared with the potential market in smaller countries.

Initially, the acute toxicology of new compounds is determined so that advice may be given to researchers conducting chemical, biological and formulation studies and, if appropriate, to make decisions with respect to further development. As the candidate proceeds through the various stages of biological evaluation, the programme of studies widens to support the development of the compound and ultimately to satisfy the regulatory authorities.

The emphasis on global markets means that studies to define the safety of candidates must comply with the requirements of all the major regulatory authorities. Detailed guidelines are produced by individual countries and by international organizations such as the World Health Organization, the FAO and the Council of Europe.

Toxicology

Toxicology studies are exercises in prediction. They are also extremely expensive and form the major component of the total development budget for a new fungicide. Consequently, tests are carried out only as they become necessary to progress a candidate towards registration.

A broad range of tests is employed, which examine the safety of new compounds in rats, mice, dogs and primates in a stepwise procedure, depending on the stage of development of the fungicide candidate. As this process is the most expensive of all development costs, the agrochemicals industry has good reason to welcome the development and acceptance of animal-free toxicology tests. However, the debate that questions the use of animals in toxicological tests has failed, so far, to produce an alternative that is acceptable to regulatory authorities.

Acute toxicology testing involves the derivation of the lowest dose resulting in 50% mortality (LD_{50}). LD_{50} values are ranked according to toxicity. Values of less than 5 mg/kg body weight (bw) are very toxic; values between 5 and 50 mg/kg bw are toxic; those between 50 and 500 mg/kg bw are harmful. The LD_{50} values for fungicides are generally high, demonstrating very low oral toxicities (Table 12.1).

Table 12.1. Acute toxicology of a range of fungicides. (Authors' own table.)

Compound	LD_{50} (rats) (mg/kg bw)
Benomyl	10,000
Captan	9,000
Chlorothalonil	10,000
Cyproconazole	1,020
Cyprodinil	2,000
Fenpiclonil	5,000
Fenpropimorph	3,000
Fentin	140–298
Iprodione	3,500
Kresoxim-methyl	5,000
Mancozeb	5,000
Metalaxyl	669
Polyoxin	21,000
Propiconazole	1,517

LD_{50}, lowest dose resulting in 50% mortality; bw, body weight.

LD_{50} values are used to design subacute studies for longer-term evaluations of toxicology. These include 90-day feeding studies and others of up to 2 years' duration which explore possible chronic, oncogenic (tumour-inducing), mutagenic and reproductive effects. The metabolic fate of the new fungicide in animals is also examined. Tests are planned strategically to coincide with nodal decision points corresponding to the maturity of other tests in the development programme (Fig. 12.1). It is current policy to review the toxicology of pesticides every 10 years.

Environment

Fungicide use is intimately involved in ecosystem dynamics and new compounds are assessed for their potential impact in a variety of environments.

Most fungicides are applied as foliar sprays. Some are used as seed treatments. Inevitably, a significant proportion of the fungicide used to control disease finds its way into the soil where it may be degraded by microbial action or through direct chemical reaction or move in the soil water and in direct runoff to water courses or to the underlying water table. Fungicides entering water courses may adversely affect aquatic life or the wildlife associated with a water environment. Likewise, fungicides may affect soil microorganisms

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or may be consumed by animals and introduced into food webs. It is necessary, therefore, that all new compounds at an appropriate stage of development are investigated with respect to their environmental fate and safety.

The first tests are straightforward, determining water solubility, lipophilicity, adsorption/desorption characteristics and hydrolytic capacity. With prior knowledge of the parameters that govern mobility of compounds in soil, reasonable predictions can be made of the potential environmental impact of the new compound. Subsequent tests probe the breakdown and metabolism of the candidate fungicide and its metabolites in soil and water.

The potential of a compound to leach is extremely important, and there is legitimate public concern about the presence of pesticides in drinking-water. Leaching studies carried out in the laboratory may overestimate the potential of a fungicide to move in soil water but are useful in comparative tests with compounds of proven mobility. The use of lysimeters is now standard practice and can provide realistic measurements of fungicide movement over extended periods in a variety of soil types. In 1980, an EU directive set the acceptable limit for individual pesticides in water at 0.1 ppb, although there is no toxicological basis for that level. Proof that fungicides are present at levels below 0.1 ppb often stretches the limits of the available analytical methods.

Lysimeter methodology, combined with the use of radio-labelled compounds, can also be used to investigate the fate of the parent and its degradation products in soils, in the presence and absence of crops. The effects of light, temperature, rainfall, moisture content, pesticide concentration and soil type in aerobic and anaerobic conditions may be determined over time and used to establish the half-life, and hence the time to 90% disappearance, of the fungicide.

Because of the possibility of runoff into water courses and, in the case of rice fungicides, the use of products in paddy environments, the toxicology of new compounds to aquatic fauna and flora is determined using fish (trout and carp), *Daphnia* and algae.

Tests on birds are routine and include both acute and chronic studies designed to mimic the effects of scavenging activity in seedling crops and at harvest. Other studies include those on beneficial insects, for example bees, earthworms and soil microorganisms. The effects of candidate fungicides are also assessed on non-target plant species (Pilling *et al.*, 1996) (Table 12.2).

Predictions of the field performance of candidate compounds in the environment are based on the accumulated data, either directly or by the use of one of the many available mathematical models, for example the leaching estimation and chemistry models (Arias-Estévez *et al.*, 2008). However, ultimately it may be necessary to confirm the results of laboratory and lysimetry experiments in field trials.

An example of the process is seen in studies using quinoxyfen which showed the parent compound to be resistant to leaching and to be stable. Metabolic products were identified in a variety of different soil types and other environmental situations. The principal compounds were 5,7-dichloro-4-(4-fluorophenoxy)-3-hydroxyquinoline (3-OH-DE-795) in soil and water/sediment tests and 2-chloro-10-fluoro(1)benzopyrano (2,3,4-de)quinoline (CFBPQ) in water and air. A minor metabolite, 5,7-dichloro-4-hydroxyquinoline (DCHQ), which formed only under acid conditions (pH 4.2) in soil and water/sediment, was judged as irrelevant to the study (Reeves *et al.*, 1996) (Fig. 12.2).

Residues

The main point of exposure of the general public to any crop pesticide is at the time of consumption of the treated crop product. For that reason, the quantity and quality of pesticide residues in the crop at harvest are determined. Additional studies on the fate of residues in cooking, baking, refining and processing, including taint testing, may be carried out.

Residue trials are conducted in field crops in a variety of environments over at least two seasons. As with crop phytotoxicity studies, residue trials employ twice the maximum optimum rate of application of the test compound. Furthermore, the potential for accumulation in meat and milk is determined. Any major metabolites of the parent compound that are discovered undergo an independent series of toxicology and environmental tests.

For example, the principal residues in wheat treated with quinoxyfen are predominantly the

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Table 12.2. Higher plants tested for azoxystrobin safety. (From Pilling *et al*., 1996, used with permission.)

Family	Species
Dicotyledons	
Amaranthaceae	*Amaranthus retroflexus* (pigweed)
Chenopodiaceae	*Beta vulgaris* (sugarbeet)
	Chenopodium album (fathen)
Compositae	*Bidens pilosa* (cobbler's pegs)
	Xanthium strumarium (cocklebur)
Convolvulaceae	*Ipomoea lacunosa* (morning glory)
Cruciferae	*Brassica napus* (oilseed rape)
Euphorbiaceae	*Euphorbia heterophylla* (spurge)
Leguminosae	*Glycine max* (soybean)
Malvaceae	*Abutilon theophrasti* (velvetleaf)
	Gossypium hirsutum (cotton)
Polygonaceae	*Polygonum aviculare* (knotgrass)
Rubiaceae	*Galium aparine* (cleavers)
Monocotyledons	
Cyperaceae	*Cyperus esculentus* (yellow nutsedge)
	Cyperus rotundus (purple nutsedge)
Gramineae	*Alopecurus myosuroides* (blackgrass)
	Avena fatua (wild oat)
	Digitaria sanguinalis (crabgrass)
	Echinochloa crus-galli (barnyard grass)
	Oryza sativa (rice)
	Setaria viridis (green foxtail)
	Sorghum halepense (johnson grass)
	Triticum aestivum (wheat)
	Zea mays (maize)

parent compound and a mixture of small-chain organic acids. Photodegradation of the parent on leaf surfaces produces a third and minor metabolite, DCHQ, which is present at much less than 0.4 mg/kg plant material. Studies on subsequent crops showed that quinoxyfen is unlikely to be taken up via the roots. It was also demonstrated that quinoxyfen was the only significant residue in edible plant tissue (Reeves *et al*., 1996).

Several immunodiagnostic assays are available for the detection of certain fungicides in food, food products and the environment. The permitted levels for most fungicides are of the order of 1–20 ppm. Diagnostic assays, based on ELISA technology, have detection capabilities to 1 ppb. More recently, mass spectrometry methods have come to the fore (Grimalt and Dehouck, 2016).

Residue levels are dependent on the agricultural systems that apply in each country. Sunlight, rainfall and temperature conditions, soil types and crop storage methods differ between each country. Hence many countries require residue testing to be carried out under local conditions.

A 2017 survey carried out by the European Food Safety Authority found that 96% of samples tested fell within legal limits. The overall risk to consumers from exposure to pesticide was considered to be low (European Food Safety Authority, 2019).

Operator safety

Operator safety is assessed in a series of experimental exposure studies carried out under practical conditions of fungicide application. In the UK, the Control of Pesticides Regulations (1986) require that persons handling pesticides, engaged in their distribution or applying them to crops are suitably qualified by validated examination. Under the EU harmonization legislation guidelines for the setting

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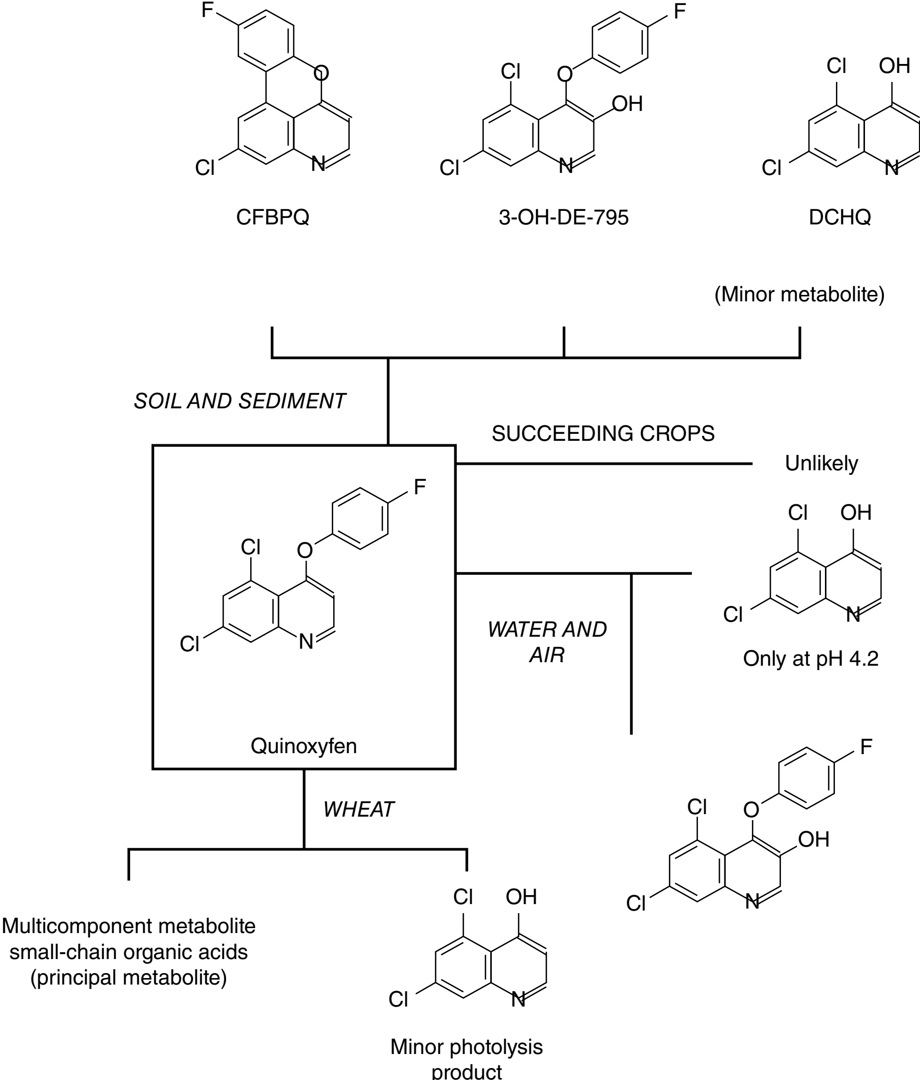

Fig. 12.2. Metabolites of quinoxyfen (CFBPQ, 2-chloro-10-fluoro(1)benzopyrano(2,3,4-de)quinoline; 3-OH-DE-795, 5,7-dichloro-4-(4-fluorophenoxy)-3-hydroxyquinoline; DCHQ, 5,7-dichloro-4-hydroxyquinoline). (From Reeves *et al*., 1996.)

and application, an *acceptable operator exposure level* (AOEL) has been established.

Long-term risks

For each candidate fungicide, the highest dose applied over the normal lifespan of test animals that causes *no observable effects* (the no observable effects limit; NOEL) is used to derive a value for the maximum *acceptable daily intake* (ADI) for a person. Using residue data and a knowledge of the daily intake of various food crops, the ADI and the toxicological characteristics of the fungicide can be compared. Only if the ADI differs from the NOEL by a factor of at least 100 is the candidate considered to present no long-term risk to consumers of treated crops (Table 12.3).

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Table 12.3. Acceptable daily intake (ADI) and no observable effect level (NOEL) for a range of fungicides. (Authors' own table.)

Compound	ADI (mg/kg bw)	NOEL (rats) (mg/kg diet)	NOEL (dogs) (mg/kg diet)
Benomyl	0.0200	2500	500
Captan	0.1000	2000	–
Chlorothalonil	0.0030	60	120
Fentin	0.0005	2	5
Iprodione	0.3000	1000	2400
Mancozeb	0.0500	–	–
Metalaxyl	0.0300	–	250
Triadimenol	0.0500	125	–
Flusilazole	0.0010	10	5
Vinclozolin	0.0700	27.1	–

bw, body weight.

In most cases, the consumption of synthetic pesticides in food is less than 10% of the ADI, even assuming an excessive intake of treated crops.

Resistance risk

It is a requirement for registration of new fungicides under EU legislation that an assessment of resistance risk, including details of a monitoring programme and baseline response data, and, if appropriate, a resistance management strategy should be supplied. It is now common practice that restrictions are placed on the number of times a product can be applied to a crop in a given season. This restriction may also be shared across all fungicides with the same MOA. Additionally, the use of mixtures of fungicides from different MOA groups can also be proscribed by the regulations.

The deregistration of actives because of safety concerns has the unintended effect of reducing options for fungicide control and hence increasing the risk of fungicide resistance.

The Label

The regulations are reflected and summarized in the 'Label'. The label can be a long and complex document. It describes which crops can be treated with the fungicide and for which diseases; when, how often and how much fungicide should be applied; and whether there are any pesticides that cannot be used in conjunction with the fungicide.

It is critical that the person applying the fungicide has a thorough understanding of the label and its conditions. Many 'labels' are detailed documents of 50 pages or more of fine print. It is the operator's responsibility to understand and apply all the conditions. The 'Directions for Use' describe the allowed uses of the product on each crop species and the target diseases. It lists the 'Rate', the amount of concentrate to be added to a given volume of water, together with warnings if this rate is exceeded. It gives the permitted application rates and the length of the 'Withholding Period', the minimum time between spraying and harvesting. The application rate, spray timing and interval and even the need for existing disease differ for each of the crop–disease combination. So whereas growers of avocados are banned from consecutive sprays, growers of grapes are directed to give two or three successive sprays. Further directions are given regarding the weather and using other products.

The EU has taken a vigorous stance on pesticide risks. It has promoted implementation of Council Directive 91/414/EEC. Moves to unify national registration requirements were designed to allow the entry and use of pesticides to all EU countries operating under the legislation (Gullino and Kuijpers, 1994; European Commission, 2022). The directive enforced a review of all existing products and recognizing the need for a balance between the essential role of pesticides

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in food production and the social and political constraints will work towards:

- removal of confidentiality of testing;
- minimal use of vertebrates in testing;
- ensuring that no unnecessary pain or suffering is caused;
- maintenance of the precedence of safety and the environment over the need to produce crop protection agents;
- ensuring that candidate pesticides can provide real benefit; and
- promotion of the principles of integrated management.

Implementation of the European directive and of comparable schemes in the USA has been subject to considerable delay and debate, which has affected the progress of new materials through to registration and has impeded the re-registration of older products.

In 1992, the Organization for Economic Co-operation and Development initiated a pesticide programme with the aims of harmonizing pesticide assessment and control procedures, speeding the process of re-registration of established products and reducing risk. The International Code of Conduct on the Distribution and Use of Pesticides was adopted in 1985 and updated in 2014 (FAO, 2014). This complex and evolving field was recently reviewed (Handford *et al.*, 2015).

The European Parliament has a philosophy on pesticide use in which it seeks to eliminate compounds that pose a particular *hazard* to the public or the environment. Previously, the evaluation process attempted to quantify the *risk* of a deleterious effect. A compound is defined as hazardous if it generates a deleterious effect at any concentration. One of the most contentious hazards is so-called ‘endocrine disruption’. Endocrine disruption is manifested as, for example, alterations in sex organ development in molluscs (Bielza *et al.*, 2008; Gisi and Leadbeater, 2010). The fungicide industry argues that the concentration of compound that causes disruption should be compared with the concentration of the compound that is likely to be found in contaminated land, water courses or food products, but this proviso is not recognized by the authorities. Furthermore, the industry argues that elimination of the pesticide might lead to increased disease losses, lower food yields and higher food prices, which might be much more damaging to the health of the population than the fungicide residue. In response to this argument, the EU has introduced the notion of ‘substitution’. This states that if a ‘hazardous’ compound could be substituted by a compound with the same or similar crop protection properties, then the hazardous compound must be withdrawn. The result of these regulations has been the wholesale withdrawal of compounds from the market. Many of these compounds were old and out of patent and the decision to withdraw was taken in some cases not because of toxicity but because the cost of maintaining registration could not be covered by future predicted sales. Hence some useful products for small markets may have been inadvertently lost. Figure 12.3 shows that the number of products available to growers in the EU has remained rather constant over the last 30 years as the introduction of about 100 new compounds (somewhere in the world) has been matched by the loss of registration of about 80 compounds and the failure to register a further 20. The loss of these compounds increases the pressure for resistance development on the remaining actives. The general rule that diversity in pesticide use prolongs the effective life is compromised if useful and safe compounds are not available for use.

The Danish government has added an extra layer of regulations designed to reduce the amount of pesticide used in its country. Around 2000, Denmark introduced a simple regulation limiting the total weight of pesticide that can be applied to fields. This straightforward but blunt measure had the effect of promoting the use of compounds with high specific activity regardless of the degree of toxicity at the permitted rate. To counter this unintended effect, the Danish authorities added a toxicity tax (Kudsk *et al.*, 2018). Under this system, the pesticide is scored for a range of toxic properties relevant to the Danish conditions. The ‘Pesticide Load’ (PL) is the sum of a PL^{HH} for human health, a PL^{eco} for ecotoxicology and a PL^{fate} for environmental fate. PL^{HH} measures the toxic load suffered by the operator when handling and applying the product, PL^{eco} measures the toxicity to animals and plants in the area surrounding the field and PL^{fate} reflects the rate of degradation in the soil and the risk of accumulation in groundwater. The PL scores are then used to set a tax for the pesticide. Hence a farmer needs

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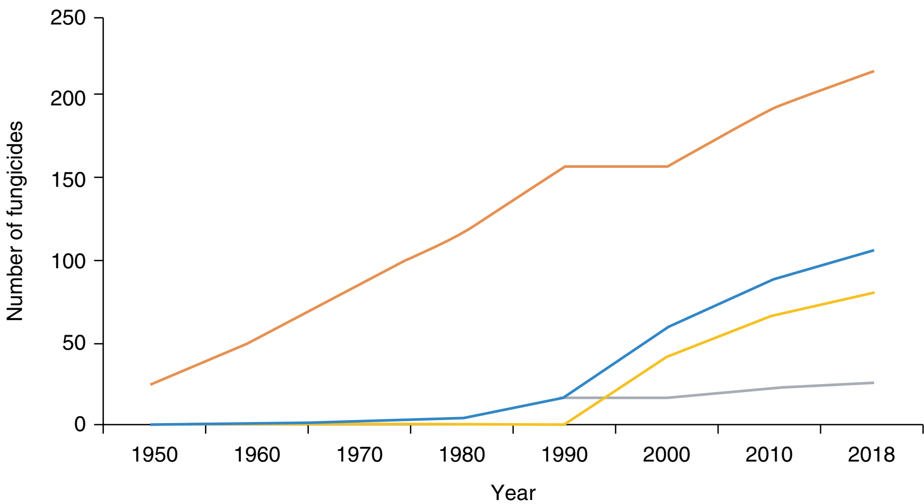

Fig. 12.3. Cumulative numbers of fungicides approved (orange line), unused (grey line), deregistered (yellow line) and unused + deregistered (blue line) in the European Union, 1950 to 2018. The available number of fungicide actives has remained rather constant at about 100. (Authors' own data.)

to weigh up the extra cost of a fungicide versus the control that a particular compound affords. For example, epoxiconazole has a much higher score for each of the PLs than prothioconazole. As a result, the pesticide tax applied to epoxiconazole products is about $25/ha (DKK 253/ha) whereas for prothioconazole the tax is about $5/ha (DKK 50/ha). Many other EU countries have imposed similar disincentives.

It is clear that the trend in pesticide legislation and registration is for ever greater stringency. We can confidently predict that registration will become ever more complex and expensive in key markets. As a result, we can predict that pesticides will continue to become safer to operators and more environmentally benign. Harmonization of legislation between different countries and supranational authorities would at least limit the burden of legislation on the industry and lead to greater efficiency for growers as well as greater reliability of food safety and supply.

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13

The Future of Disease Control

Key Points

- The incessant rise in food demand means that all reliable methods of crop protection must be deployed at full efficiency and in an integrated manner.
- Global warming and biosecurity failures are likely to further impact crop protection.
- Many existing fungicides are likely to be phased out due to regulatory challenges.
- The evolution of fungicide resistance means that resistance management strategies must be deployed to extend the useful life of existing actives.
- There is an ongoing need for new actives with new MOAs. The pipeline for new actives is working but at ever-increasing cost. Genomics and molecular modelling are likely to have an increasing impact.
- New approaches in fungicide development and disease control include RNA-based fungicides (e.g. RNAi); genomic approaches and molecular modelling; and transgenic and gene-edited plants that improve or modify disease resistance.
- Developing genetically modified (GM or GE) traits to replace or more likely supplement fungicides will require a major shift in public perception.

Food Demand and Disease Threats

The world's population is growing at a faster pace than ever before and looks set to increase until at least 2050. The population needs to be fed and needs somewhere to live. Hence more food needs to be grown on less land with less water. To reduce the levels of food insecurity that already exist in parts of the world and to prevent food deficits occurring in more productive regions, efficient and effective methods of crop production must be introduced and maintained.

There are many reasons to believe that the disease pressure on crops will increase. Global warming will have varied and rather unpredictable effects on crop diseases (Carlton *et al.*, 2012; Fisher *et al.*, 2012; West *et al.*, 2012) but generally will decrease food security. Global warming and ever-increasing international travel and trade will reduce or even eliminate the power of national quarantine agencies to keep exotic pathogens out of their countries. History teaches that plant pathogenic fungi and oomycetes will always challenge our ability to produce food in quantity and of an acceptable quality. Pathogens evolve to overcome genetic disease genes as well as fungicides. If diseases are well controlled, new pathogens have emerged to take advantage of the reduced competition.

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Although the introduction of monocultures provided crop pathogens with an ideal environment in which to multiply, the situation in some crops was exacerbated by techniques that were subsequently adopted to manage other problems. In cereals, the drive to increase yield through improved varieties and higher fertilizer inputs highlighted the value of good weed control. The ensuing spiral towards higher yields through the increasing use of fertilizers and herbicides eventually hit the yield-limiting factor of plant disease. Fungicides allowed yet more fertilizer to be used, to achieve even greater yields.

The effects of crop disease cannot be trivialized because they are never far away. Current estimates suggest that without fungicides we would lose up to one-third of yield, depending on the crop. In some circumstances, total loss is possible. This reality necessitates the use of crop protection management systems that contain fungicides as an integral component.

The development and use of fungicides in crop protection is a success story. It is a story that has developed from their earliest and crude application in agriculture and horticulture, through a series of technological evolutionary steps, to a point where products are able to exert safe, broad-spectrum control for extended periods, or to work precisely to protect against attack by specific pathogens, or even to influence the host itself to combat infection. However, the process of improvement in crop disease management continues and the next 20 years are likely to witness even greater changes in fungicide technology and use.

Loss of Existing Fungicides

We have already seen that regulations vigorously initiated in Europe have led to the withdrawal of many active compounds. Many other countries follow the lead of Europe either because they accept the findings of the EU agencies or because they wish to continue exporting to the EU. The ever-tightening regulatory demands have increased the pressure on the remaining compounds as growers have a restricted range of products at their disposal. The DMI group is already under serious threat and its loss could have a massive impact on the quantity and quality of food production worldwide.

Fungicide resistance preceded the withdrawal of the MBC class of fungicides by some years. Other fungicides afflicted significantly by resistance, including the DMI, QoI, PA, CAA and SDHI groups, remain in use. Indeed, predictions that QoIs would become useless through resistance have proved very wide of the mark. Instead, fungicide resistance management strategies have ensured their continued use. The strategies involved mixtures and alternations of fungicides. Hence there is a strong demand for new fungicides to fulfil roles in resistance management.

The Discovery Process

The pace of fungicide discovery shows no clear sign of slowing up, but the process is proving to be increasingly complex and expensive. The discovery and development of new fungicides is almost exclusively the province of private-sector companies with only a handful still active in this demanding endeavour. Public-sector support for synthetic fungicide discovery is limited to upstream research. Many companies are also turning to biological fungicides as these are much cheaper to discover and register, and this activity is supported to a significant extent by public-sector investment.

None the less, the low-hanging fruit have been picked. The unique biomolecules in fungi, particularly the ergosterol biosynthesis pathway, have been thoroughly examined for fungicide targets. It seems inevitable that newer fungicides will require a more expensive discovery pathway than existing ones.

Genomics has not yet had a profound impact on the processes of fungicide discovery. However, we now have the situation in which the genome sequences of all relevant organisms, including the target oomycete and fungal pathogens, the host crops and key off-target organisms, have been obtained for at least one isolate and in most cases for many. The 'pan-genome' is the term used to describe all the genomic variability of a species. It is therefore possible to imagine a genomics-led discovery process in which molecules will be designed to bind and inhibit

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key enzymes in pathogens only and have no effect on non-target organisms. This is theoretically straightforward, but such a development will require a sustained effort in genomics and automated protein structure prediction.

RNA-based fungicides; spray-induced gene silencing

The central dogma of molecular biology states that DNA makes RNA makes protein in a linear fashion. Various strands of research over the last 20 years have highlighted exceptions to this whereby RNA molecules inhibit the translation of gene transcripts, a process known generally as RNA interference or RNAi (Cai *et al.*, 2018). The mRNA is targeted by a short RNA molecule that is complementary in sequence. This creates a short stretch of double-stranded RNA (dsRNA). dsRNA is efficiently detected by a set of enzymes that cleave the RNA and inactivate it before it is translated into proteins. There are two pathways for exploitation of this phenomenon: (i) expression in plants of GM RNAi constructs, this is known as host-induced gene silencing (HIGS) (Nunes and Dean, 2012); and (ii) RNA molecules can be directly delivered into fungi on crops, a technology known as spray-induced gene silencing (SIGS). HIGS represents a GM strategy and suffers from the public reluctance to accept this technology. SIGS, on the other hand, represents an entirely new paradigm for external control of diseases (McLoughlin *et al.*, 2018; Sang and Kim, 2020).

RNA is a promising class of molecule for disease control. It is a naturally occurring molecule that is broken down by enzymes found in all organisms. It should therefore have a high degree of inherent safety and low degree of environmental persistence. Its inhibitory effect is a direct consequence of the sequence of bases in the RNA molecule and this means that even very short RNA sequences can be designed that are unique. It should be possible therefore to use the rapidly growing database of genome sequences to design molecules that have the desired degree of specificity to target all relevant pathogens but leave non-target organism unaffected. The evolution of resistance to these new RNA fungicides is an issue that has generated much speculation. If the inhibitory effect requires a perfect match between the RNAi and the mRNA, it would be expected that only a single base-pair change in the target gene would be needed to render the RNAi ineffective. And unlike target site modifications that affect conventional chemical fungicides, even synonymous mutations not affecting the amino acid sequence would be resistant. This ease of resistance could theoretically be countered by employing a mixture of RNA sequences using the same logic that applies to conventional chemicals. So far, no RNA fungicides have progressed far down the development pipeline although it is clear that much research is going on in industry and academic laboratories. The chemical synthesis of RNA-based molecules is currently very expensive, but the rise of RNA-based human vaccines may reduce costs.

Genetic Disease Control

Molecular plant breeding allows breeders to combine in one cultivar all the best alleles of disease resistance genes as well as other desirable traits, as long as markers for the genes of interest have been discovered. This process has not progressed as fast as was predicted and, to date, only major resistance gene markers are in general use. The quantitative and minor genes typical of so many resistance phenotypes have been harder to pin down. Developing the understanding of pathogenicity mechanisms in more fungi and better genomic resources for more crops will accelerate this process.

Mixtures of cultivars

Most crops are grown as monocultures of one cultivar of a single crop. There are clear advantages to a farmer in growing a monoculture culminating in the production of a crop optimized for a single use. It has long been discussed that monocultures represent a perfect environment for a pathogen as every plant will be equally susceptible. Theoretical considerations have long predicted that mixtures of cultivars with different disease resistance characteristics will suffer less disease damage. The issue has been whether the advantages in terms of disease control of a

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crop cultivar mixture can outweigh the production and marketing disadvantages. A recent meta-analysis indicates that mixes can work for feed wheat production and help limit losses to SEPTTR (Kristoffersen *et al*., 2020). Results will doubtless vary between different crops and uses but it remains a valid subject for investigation.

Transgenic (GM) disease control

Mechanisms that permit the transfer of alien genes into plants have been available for over 35 years. Nearly all crop species can be transformed at least in some cultivars and the methods used are generally quite inexpensive. Intellectual property issues associated with GM methods remain significant but are receding as the patents expire.

Starting in the 1980s when GM technologies were new and deemed to be technically risky, the commercial exploitation of the technology was undertaken by new sections developed within or acquired by the existing chemical companies. Indeed, many chemical companies bought seed companies to have a route to market the new disease resistance traits. They pursued only the biggest markets with the greatest profit potential. Hence the great majority of GM crops released to date involve genes for herbicide resistance and for insect tolerance.

Resistance to diseases has been under study in academic as well as industrial laboratories for some time. As long ago as 1991, it was shown that the expression of alien genes controlling hydrolytic enzyme activity in transgenic tobacco and oilseed rape resulted in increased resistance to infection by *Rhizoctonia solani* (Broglie *et al*., 1991). A great deal has been learnt about how pathogens cause disease and how plants resist infections, and a good deal of this information has been directed towards the creation of GM disease-resistant crops (van Esse *et al*., 2020). Many experiments have successfully generated disease resistance crop lines. However, the testing of such plants has been limited to tightly regulated environments, whether growth chambers, glasshouses or field stations. To date, no commercial crops with transgenic fungal or oomycete disease resistance have been released.

The reasons for this glaring failure are partly scientific but mainly political. Developing a GM disease resistance trait is beset with many of the same difficulties as developing a new fungicide; the GM trait should generate good levels of disease resistance against a wide spectrum of pathogens and should be safe. Research was carried out on a wide scale in both university and chemical company laboratories. The scientific questions are tough but surely would have been solved had the level of investment present through the 1980s and 1990s been maintained. The backlash against GM products that emerged in Europe in 1996 following the 'mad cow disease' outbreaks caused both public- and private-sector organizations to cut back investments in this area. GM herbicide- and insect-resistant crops have been grown on a huge area and no deleterious effects have been reported. None the less, no relaxation of the regulations has been forthcoming for genetically modified organisms (GMOs), especially in Europe. This has effectively stymied much of the research in this area. Some privately funded laboratories continue to work on this, and it is possible that their results may breach the dam and force a rethink in commercial and public-sector research environments.

Genome editing

Another possible breach in the dam might emerge from genome editing (GE). The ability to alter the genome sequence of a plant in a controlled manner has emerged in the last few years. ZFNs (zinc-finger nucleases) and TALENs (transcription activator-like effector nucleases) have been in use for several years but are relatively cumbersome in practice (Joung and Sander, 2013). It is widely predicted that CRISPR–Cas (clustered regularly interspaced short palindromic repeats) will prove revolutionary for crop disease control (Shan *et al*., 2013; Ji *et al*., 2015; Zhang *et al*., 2016) because it offers a generic and rather inexpensive method to alter and inactivate genes in all crop species. Two main targets are under study. Classical disease resistance genes can be altered to broaden the range of pathogens that they can detect. Second, many crop genes that confer susceptibility to pathogens have been discovered and these are obvious candidates for disruption by GE techniques.

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As GE techniques do not leave foreign nucleic acid sequences in the engineered plant, they do not conform with the classical definition of a GMO. Regulatory authorities have differed in their response to this new technology. The USA excluded GE techniques from GMO regulations, giving a green light to this research area. French authorities, on the other hand, argued that GE techniques were a form of mutagenesis and so brought the previously unregulated crop improvement techniques of chemical and radiation mutagenesis into the same category as GMO. The rest of Europe is still debating.

The Future

How will disease control change in the next decades? The most likely scenario is that it will not change very much. Pathogens will continue to spread to new areas despite the best efforts of biosecurity and quarantine agencies. Climate change is likely to increase the impact of pathogens as they arrive in new areas. Fungicide resistance will spread across national boundaries.

However, new specific actives continue to be developed and marketed. New QoI and DMI actives have recently been released as well as fenpicoxamid, a new QiI. The pipeline of new actives is working even if it is proving more difficult and expensive than before. New technologies such as RNAi could well have an impact. However it seems that as fungicides become more active, safe and specific, they also become more prone to suffering from resistance. Methods to improve the efficacy and range of BCAs will hopefully emerge. Plant breeders are getting better at generating resistant lines that combine efficacy with robustness. GM and GE techniques, perhaps combined with an exogenous active, will surely fulfil the long-held promise.

Overall, the struggle to protect crops from disease will continue. Current losses of 20% are not sustainable as the world's population peaks and meat consumption increases. The one certainty is that the ingenuity of the plant protection community will be tested as never before.

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Appendix – EPPO Codes

Abbreviation (EPPO code)	Name of pathogen	Host/s and disease/s
ALTEAL	*Alternaria alternata*	Tobacco brown spot and many others
ALTEBI	*Alternaria brassicicola*	Black spot of crucifers
ALTELO	*Alternaria longipes*	Brown spot of tobacco
ALTELY	*Alternaria arborescens*	Stem canker of tomato
ALTESO	*Alternaria solani*	Potato (tomato) early blight
ALTETO	*Alternaria tenuissima*	Tomato nail-head spot
ASPEFL	*Aspergillus flavus*	Mycotoxigenic spoilage organism
ASPEFU	*Aspergillus fumigatus*	Anthracnose of strawberry
ASPEND	*Aspergillus nidulans*	Model fungus
ASPEPA	*Aspergillus parasiticus*	Mycotoxigenic spoilage organism
BOTRCI	*Botrytis cinerea*	Botrytis grey mould
BOTREL	*Botrytis elliptica*	Lily grey mould
CANDAL	*Candida albicans*	Human pathogen
CERCBE	*Cercospora beticola*	Sugarbeet leaf spot
CERCKI	*Cercospora kikuchii*	Cercospora leaf blight
CERCZN	*Cercospora zeina*	Maize grey leaf spot
CLADCA	*Venturia effusa*	Pecan scab
COCHCA	*Cochliobolus carbonum*	Northern corn (maize) leaf blight
COCHHE	*Cochliobolus heterostrophus*	Southern corn (maize) leaf blight
COCHME	*Cochliobolus miyabeanus*	Rice brown spot
COCHSA	*Cochliobolus sativus*	Wheat and barley spot blotch
COLLAC	*Colletotrichum acutatum*	Anthracnose of strawberry
COLLDU	*Colletotrichum truncatum*	Anthracnose of capsicum, etc.
COLLGR	*Colletotrichum graminicola*	Maize anthracnose
COLLGL	*Colletotrichum gloeosporioides*	Broad host range anthracnose
COLLLA	*Colletotrichum lagenarium*	Cucumber anthracnose
CORYCA	*Corynespora cassiicola*	Cucurbit blotch
DIDYBR	*Stagonosporopsis cucurbitacearum*	Black rot, gummy stem of cucurbits
ERYSGH	*Blumeria graminis* f. sp. *hordei*	Barley powdery mildew
ERYSGT	*Blumeria graminis* f. sp. *tritici*	Wheat powdery mildew
EUROOR	*Aspergillus oryzae*	Model organism

Continued

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Continued.

Abbreviation (EPPO code)	Name of pathogen	Host/s and disease/s
FILBNF	*Cryptococcus neoformans*	Human pathogen
FULVFU	*Cladosporium fulvum*	Tomato leaf mould
FUSAAZ	*Fusarium asiaticum*	Wheat fusarium head blight
FUSAME	*Fusarium oxysporum* f. sp. *melonis*	Fusarium wilt of melon
FUSASO	*Fusarium solani*	Many bulb and root rots
FUSAOX	*Fusarium oxysporum*	Many wilts and root rots
FUSAPF	*Fusarium proliferatum*	Soybean root rot
FUSAVR	*Fusarium verticillioides*	Maize and cotton wilt and rots
GIBBFU	*Fusarium fujikuroi*	Rice bakanae disease
GIBBZE	*Fusarium graminearum*	Wheat and barley Fusarium head blight
HELMSO	*Helminthosporium solani*	Potato silver scurf
LEPTMA	*Plenodomus lingam*	Blackleg or phoma
LEPTNO	*Parastagonospora nodorum*	Wheat septoria nodorum blotch
MICDMA	*Microdochium majus*	Cereal snow mould
MONGNI	*Microdochium nivale*	Cereal snow mould
MONIFC	*Monilinia fructicola*	Stone fruit brown rot
MONILA	*Monilinia laxa*	Stone fruit blossom blight
MYCOFI	*Mycosphaerella fijiensis*	Banana black sigatoka
MYCORA	*Didymella rabiei*	Chickpea blight
NEUSCR	*Neurospora crassa*	Model organism
PENIAU	*Penicillium aurantiogriseum*	Spoilage pathogen of fruits
PENIEX	*Penicillium expansum*	Apple blue mould
PENIIT	*Penicillium italicum*	Citrus blue mould
PENIDI	*Penicillium digitatum*	Citrus green mould
PHAKPA	*Phakopsora pachyrhizi*	Asian soyabean rust
PHYTCP	*Phytophthora capsici*	Pepper blight
PHYTDR	*Phytophthora drechsleri*	Watermelon fruit rot
PHYTIN	*Phytophthora infestans*	Potato (tomato) late blight
PHYTMS	*Phytophthora sojae*	Soybean root rot
PHYTNN	*Phytophthora nicotianae* var. *nicotianae*	Black shank of tobacco
PLADBR	*Plasmodiophora brassicae*	Clubroot
PLASVI	*Plasmopara viticola*	Grapevine downy mildew
PLEOAL	*Stemphylium vesicarium*	Onion leaf blight
PODOFU	*Podosphaera fusca*	Bean powdery mildew
PODOLE	*Podosphaera leucotricha*	Apple powdery mildew
PODOXA	*Podosphaera xanthii*	Cucurbit powdery mildew
PSDCHA	*Oculimacula acuformis*	Wheat eyespot
PSDCHE	*Oculimacula yallundae*	Wheat eyespot
PSPECU	*Pseudoperonospora cubensis*	Cucurbit downy mildew
PUCCGT	*Puccinia graminis* f. sp. *tritici*	Wheat stem rust
PUCCHD	*Puccinia hordei*	Barley leaf rust
PUCCRT	*Puccinia triticina*	Wheat leaf rust
PUCCSO	*Puccinia sorghi*	Maize common rust
PUCCST	*Puccinia striiformis*	Wheat yellow rust
PYRIOR	*Magnaporthe grisea*	Rice (wheat) blast
PYRNTE	*Pyrenophora teres*	Barley net blotch
PYRNTR	*Pyrenophora tritici-repentis*	Wheat tan spot
PYRPBR	*Pyrenopeziza brassicae*	Canola light leaf spot
PYTHSP	*Pythium* spp.	Damping-off disease
RAMUCC	*Ramularia collo-cygni*	Barley ramularia blotch

Continued

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Continued.

Abbreviation (EPPO code)	Name of pathogen	Host/s and disease/s
RHIZCE	*Rhizoctonia cerealis*	Sharp eye spot and bare patch of cereals
RHIZSO	*Rhizoctonia solani*	Damping-off (many hosts)
RHYNSE	*Rhynchosporium secalis*	Barley scald
SACCCE	*Saccharomyces cerevisiae*	Model fungus
SCLEHO	*Clarireedia homoeocarpa*	Turfgrass dollar spot
SCLESC	*Sclerotinia sclerotiorum*	Sclerotinia stem rot (many hosts)
SCPHMA	*Sclerophthora macrospora*	Maize downy mildew
SEPTTR	*Zymoseptoria tritici*	Wheat septoria tritici blotch
UNCINE	*Erysiphe necator*	Grapevine powdery mildew
USTIMA	*Ustilago maydis*	Maize smut
USTNVI	*Villosiclava virens*	Rice false smut
VENTIN	*Venturia inaequalis*	Apple scab

EPPO, European and Mediterranean Plant Protection Organization.

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Fungicides in Practice

Richard P. Oliver and Janna L. Beckerman

This is an up-to-date guide on the science and practice of disease control based on fungicides in horticulture and broad acre agriculture. It describes how conventional, organic and biological fungicides are discovered, how they work and how resistance evolves. Chapters on formulation, mode of action, mobility and application inform decisions about which fungicides to use, when to use them, and how to rotate (or tank-mix) them, to manage both plant disease and fungicide resistance. A chapter on experimental design of fungicide trials aids practitioners in designing their own trials to evaluate how effective products are for their plant disease problem.

Based on the successful 2014 book, *Fungicides in Crop Protection*, this edition has four entirely new chapters, and extensive updates to the other nine chapters.

The contents include:

- Fungicide markets, discovery and performance
- Modes of action and spectrum
- Biological crop protection, and organic cultivation
- Fungicide formulation, mobility and application
- Experimental design of fungicide trials and their analysis
- Fungicide resistance
- Legislation and regulation

Written for crop protection professionals and scientists, growers, agronomists and consultants, the book is also suitable for students of agriculture and agronomy.

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