4 Fungicide Discovery

Key Points

- Strategies to discover new fungicides focus on:
  - diseases with the greatest market potential;
  - new and emergent diseases;
  - novel MOAs; and
  - experimentally tractable pathogens.
- 'Leads' are active compounds with the potential to be modified to optimize 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

There are many thousands of species of plant pathogenic fungi. Fortunately, for each crop the array of fungi able to attack, colonize and cause damage is limited. Those that are successful can result in significant economic losses under suitable environmental conditions. In wheat, for example, over 15 pathogens are recognized to cause 19 distinct and severe disease syndromes in Australia alone (Murray and Brennan, 2009). They include biotroph and necrotroph, foliar, root, crown and seed pathogens, and both *Basidiomycota* and *Ascomycota*. In the case of grapevines, the three major pathogens include two ascomycetes and one oomycete (Essling and Francis, 2012).

To a grower, all pathogens that affect yield or quality enough to cause a reduction in profit are important and appear to constitute worthwhile targets for fungicide discovery. However, fungicide manufacturers will invest research and discovery resources only in the control of those pathogens which have the capacity to return an acceptable profit, and the development of fungicides into niche markets is always preceded by their success in major markets.

The questions, then, are how are those major targets distinguished from the plethora of possible crops and their attendant diseases and how are they incorporated into discovery research programmes? The choice of fungicide targets has been driven by a combination of factors. The crop should be widely grown and/or should be of high value. Such crops should also have diseases that are not well controlled by genetic or cultural methods.

Each agrochemicals 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 novel and 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 potentially 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 particular crops.

Definition of target pathogens and crops

Market size

Before planning the development of any new fungicide, manufacturers must be convinced that the product will reach a threshold level of annual sales in order to justify investment, sustain further research, return a profit for the shareholders, maintain a motivated and expert staff and support the future expansion of the company. The threshold varies according to company and target, but may be as high as US$200 million per annum at product maturity. The value of particular crop fungicide markets can be disassembled into the value of control associated with individual pathogens or combinations of pathogens. This process prioritizes existing and commercially proven fungicide targets.

New and re-emergent diseases

The single most critical factor in disease target choice is the economic level of damage caused by the pathogen. Changing market conditions and new knowledge can significantly alter the loss levels. Plant pathogens continually surprise us with their ability to cross borders and to take advantage of new opportunities. These new opportunities arise from a variety of factors. SEPTRI has become the dominant pathogen on cereals in Europe, displacing rusts, mildews and LEPTNO since the 1960s. The reasons for this shift in pathogen is unclear but are linked to reductions in pollution from coal-fired power stations and domestic heating (Bearchell *et al.*, 2005). Hence, SEPTRI has become the number one target for fungicide development. *Fusarium graminearum* causes the disease head blight of cereals (Kazan *et al.*, 2012). The pathogen causes only modest yield losses but contaminates infected grain with mycotoxins. The mycotoxins are extremely toxic to humans and animals and thus make the grain essentially unsellable. Therefore *F. graminearum* has become a more significant target.

One of the most important considerations is the area grown to a crop and the density with which it is planted. Both factors favour the development of diseases. A key example here is soybean, of which 250 million Mt is now grown annually on about 100 Mha. This increase in area has been driven by the need for protein to add to animal feed and human food products. A newly important disease, soybean rust or Asian rust, caused by *Phakopsora pachyrhizi*, emerged in the 2000s in South America (Yorinori *et al.*, 2005).

Soon it spread to North America (Schneider *et al.*, 2005). Fungicides to control the disease were needed urgently and were used on a large area. Over US$500 million was spent. Hence soybean rust emerged as a major target for fungicide discovery.

Potato late blight (PHYTIN) has been a major disease since the 1850s but was well controlled by genetics in Europe until recently. Genetic disease control can be compromised if the pathogen can evolves new virulences faster than plant breeders can breed new resistances. Despite quarantine regulations, the A2 mating type gene emerged in Europe in the 1980s and spread throughout the continent (Fry, 1991; Dyer *et al.*, 1993). This allowed the shuffling of virulence genes and hence the defeat of the resistance genes in the major cultivars. Fungicides then had to be applied in ever-increasing frequency. And hence PHYTIN became a major priority for fungicide discovery.

Fungicide resistance

Fungicide resistance has become one of the dominant factors in target choice. Pathogens differ in their propensity to develop resistance. The pathogens that typically develop resistance first are the powdery mildews followed by BOTCIN (see Chapter 6 for details). For this reason, it is still economic to develop narrow-spectrum compounds that are specific for these pathogens (especially wheat, barley and grapevine). Recent examples include quinoxyfen, metrafenone, bupirimate, proquinazid, spiroxamine, cyflufenamid and, for BOTCIN, fenhexamid and iprodione.

The importance of fungicide resistance has placed a premium on compounds that either would 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 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.

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 are 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. It has reduced, but not entirely eliminated, the practice of seeking derivatives of existing compounds; witness the case of prothioconazole, the first new triazole to be released for 15 years.

Market deconvolution

In crops that are host to many pathogens, as in cereals, the actual value that a grower places on the control of specific pathogens is more difficult to unravel because established products are either broad-spectrum, including primary and secondary targets, or are

specific to the major targets. In those cases, it is important to determine the true values attached to the control of the component pathogens and to particular combinations. We can illustrate how a fungicide market is constructed and how disease targets are prioritized by considering the utilization of products in Great Britain in 1994. In 1994 there were 4.75 million ha of arable crops (Anon., 1994) and fungicides were applied to 67% (3.2 million ha), with, on average, two applications using three products and a total of four active ingredients. The frequency of fungicide input varied according to crop. Fungicides were used in almost all potatoes, whereas only 4.5% of the linseed crop was treated. In total, 10.8 million ha received a fungicide treatment ('treated hectares').

In cereals, with a total area of 2.8 million ha, there were 8.6 million treated ha and the percentage of area treated varied from 71.1% in spring barley to 96.3% in winter wheat (Table 4.1).

Areas were treated with fungicide according to the occurrence and severity of particular diseases. These changed from year to year but were generally dominated by the SEPRTI diseases and powdery mildews. In contrast, rust was less damaging, being severe once in about every 7 years, or only locally severe as in the occurrence of *Puccinia striiformis* in the south-eastern part of Britain.

In winter wheat, most fungicides (excluding seed treatments) were applied at two application timings, stem extension/first node (growth stage 30/31) and flag leaf emergence (growth stage 37). Different pathogen combinations were associated with each timing. In general, the first timing targeted stem base and the early foliar pathogens, eyespot, ERYSGT and SEPTRI. The second timing targeted ERYSGT, SEPTRI, *Puccinia* spp. and LEPTNO, and accounted for the bulk of foliar fungicide use. Based on this split in the chronology of disease incidence and control, figures for treated area with respect to each pathogen can be derived for each application.

In winter barley, two fungicide applications were also used but were of equal merit. Here the major pathogens, as seen by the grower, were eyespot, ERYSGH, *Pyrenophora teres*, *Rhynchosporium secalis* and *Puccinia hordei*. In spring barley, it was usual for only a single fungicide application to be made, in this case for the control of ERYSGH and *R. secalis*.

Fungicide applications cost the equivalent of US$10–50/ha. Combining treated hectares, targets and timing shows that SEPTRI control was the most valuable target for UK fungicides (US$260 million), closely followed by a collective value for wheat and barley powdery mildew (US$240 million). Eyespot and *R. secalis* control were approximately equivalent at US$50 million and US$60 million, respectively, with rust and net blotch control accounting respectively for only 3% and 0.7% of the total UK cereal fungicide market (US$750 million).

Table 4.1. Cereal fungicide use in Great Britain, 1994.

	Wheat	Winter barley	Spring barley	Total arable crops
Area planted (ha)	1,802,191	620,132	450,596	4,756,116
Treated hectares[a]	6,525,831	1,497,801	619,420	10,793,721
Percentage of area treated	96.3	93.8	71.1	

[a]Excluding seed treatments.

The value of the control of multiple cereal pathogens can therefore be estimated (Table 4.2).

A similar exercise can be carried out using the existing fungicide sales value attached to the control of pathogens of major crops in East Asia, South-east Asia, Australia, New Zealand, the Pacific Islands and South America (Table 4.3). In this example, the control of PYRIOR, with a value of US$600 million in Asia alone, is enough to qualify it as a suitable target for discovery investment. On a global basis, *R. secalis* may also be included as a primary target for fungicide discovery.

In pome fruit, the major targets arc VENTIN and *P. leucotricha*. Although activity against VENTIN is preferred, the use of programme spraying and the existing availability of broad-spectrum compounds mean that dual activity is an advantage.

This system of target definition does not accommodate those pathogens, such as BOTCIN or *Rhizoctonia*, which attack a range of different crop species. It is probable

Table 4.2. Estimated current value of fungicide targets – cereals.

Target	Potential fungicide sales (US$ million)
SEPTRI	1250
ERYSGH	950
Eyespot	180
Rhynchosporium secalis	110
Rusts	82
Pyrenophora spp.	12.5

Table 4.3. Estimated current market sizes for selected diseases in Asia and Australasia and in South America.

Pathogen	Asian + Australasian market (US$ million)	South American market (US$ million)
Soybean rust	?	500
Alternaria	125	100
BOTCIN	60	12
Cercospora	75	12
Powdery mildews	105	25
Gibberella	75	
Glomerella	125	
MYCFIJ	20	120
Penicillium	45	
Peronospora	25	
PHYTIN	100	100
Plasmodiophora	30	
PLASVIT	33	10
Eyespot	1	
Puccinia	32	18
PYRIOR	600	25
Rhizoctonia	250	0.5
Rhynchosporium	2.5	
Sclerotinia	45	
VENTIN	140	20

that the most prevalent fungal pathogen on that basis is *Cercospora*. However, no company regards the control of *Cercospora* as a primary target for discovery investment because of its fragmented spectrum across comparatively low-value crops. In practice, the definition of discovery fungicide targets is a balance between their potential global value and the number of economically important crops in which they occur. Although vegetables constitute the largest fungicide market, their diversity means they are less significant than cereals, rice, pome fruit or grapevine.

In general, companies share the same major objectives within existing markets, although some may place greater reliance upon research into the control of particular pathogens such as PLASVIT if they consider the risk–benefit balance to be favourable.

Exploratory targets

Markets are created by fungicides which demonstrate that disease control can be profitable to the grower. There are several pathogens for which no effective fungicidal control exists but which are associated with severe crop losses. Examples are *Sclerotinia* in legumes and take-all in cereals. However, it is difficult to assess with much certainty the commercial value of a fungicide that could control new target pathogens. The problem lies in the puzzle of how to compare a healthy crop against a diseased crop when no disease control exists. In addition, the control of root and crown pathogens requires that the fungicide has phloem mobility, a property that has only rarely been observed and which represents a considerable technical problem.

The investment of research funds into the discovery of compounds for new markets is risky and tends to be a second priority to finding materials to fit existing outlets. The justification to pursue some targets is growing, however (see, for example, Case Study 1).

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.

The design of fungicide screens

Screens used by fungicide companies can be divided into three broad classes referred to as 'high-throughput', *in vitro* and *in planta* (Table 4.4). These types of screen 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.

Case Study 1. The control of *Gaeumannomyces graminis* var. *tritici* – an unmet need?

Gaeumannomyces graminis var. *tritici* is the causal organism of take-all disease of cereals, a name first applied to a devastating incidence of the disease in Australia about 150 years ago. It is an ascomycete and one of the four members of the genus *Gaeumannomyces* that are known to infect the roots of grasses, including cereals and sedges. The main commercially important hosts of *G. graminis* var. *tritici* are wheat and barley, although rye is also susceptible. Infected roots are stunted and blackened, with similar symptoms occasionally extending to the stem base. Plants ripen prematurely and produce bleached ears, commonly known as whiteheads, which produce little or no grain. Following harvest, the fungus survives on stubble and the root remains of the infected crop. Volunteer plants are susceptible to attack and serve to carry infection through to the following year. In the absence of a suitable host, *G. graminis* var. *tritici* survives on crop debris in the soil as a weakly competitive saprophyte.

The pathogen is widely distributed and occurs wherever cereals are grown commercially. It is recognized to be an important determinant of yield in Australia, the Pacific north-west of America, South America and Europe. However, losses attributed solely to *G. graminis* var. *tritici* are difficult to assess because of year-to-year and between-site variations in disease incidence and yield response to infection. Disease incidence is determined mainly by:

- the proportion of susceptible crops in rotations;
- soil type; and
- soil moisture content.

However, it can be profoundly affected by other factors, such as:

- sowing date;
- cultivation practice;
- soil nutrient composition; and
- fertilizer application.

Yield losses in the Pacific north-west of America are estimated to be 10–50%. In moderate/high-risk areas of Western Australia, take-all accounts for losses of up to 40%. In England and Wales, recent estimates are for losses between 1 and 4% in second and subsequent wheat crops, although some workers regard this as conservative.

Yield losses cannot be determined accurately but, in the UK, the contribution of take-all to total loss was approximately one-sixth as great as all the other leaf and stem base diseases combined, or up to US$85 million annually (Hornby and Bateman, 1991; Yarham, 1995).

Take-all can be controlled to a degree by altering farming practice:

- lowering the inoculum levels by growing non-susceptible crops as a rotational break;
- the use of more tolerant cultivars of wheat;
- delayed sowing; and
- carefully planned fertilizer use.

More direct control measures are not practical, but the potential for fungicide use or biological control has been explored. The take-all decline syndrome is a demonstration of biological control, albeit a natural corollary to long-term wheat culture. The accumulation of antagonists by growing a suitable preceding crop, for example grass, can

Continued

Case Study 1. Continued.

delay the onset of the disease. Otherwise, bacterial antagonists such as *Bacillus* or pseudomonads can be applied to the rhizosphere directly or through seed treatment. Most claims for the success of biological control come from the USA and Australia, and several patents have been filed. However, improvements in yield have been demonstrated in only 60% of the treated crops and, at that level of success, the practical use of biological agents to control take-all is too unreliable to be commercial. Synthetic fungicides, regarded as a less environmentally friendly alternative to biological control, have had little more success in controlling the incidence of take-all. It is clear that activity against *G. graminis* var. *tritici* is not uncommon or new. The efficacy of the C14-demethylation inhibitors triadimenol and flutriafol is well documented but earlier examples, such as the pyrimidine nuarimol, demonstrate that compounds with very high activity have been available for many years. However, the failure of these materials to be developed as products for take-all control reflects the distinction between activity and performance and the difficulty in transferring *in vitro* or glasshouse *in vivo* efficacy to utility in the field. In all cases, the underlying problem is one of delivery of the active fungicide to the site of infection. Several strategies have been considered.

Soil fumigation

Soil fumigants are difficult to use on a large scale and are expensive. They have the added disadvantage of being non-selective, raising the potential problem of a subsequent rapid build-up of take-all due to the depletion of natural antagonists.

Soil fungicides

The immediate problem of using soil-incorporated fungicides to control take-all is the dilution effect of the soil on the applied product. Compounds would have to be delivered in large quantity, probably in a granular formulation, or be extremely active against *G. graminis* var. *tritici*. Assuming a recommended rate of fungicide application of 100 g of active ingredient (a.i.) per hectare and complete mixing in the soil, the fungicide concentration would decline to negligible levels by 30 cm.

Most fungicides demonstrate their highest activity *in vitro*, but few are active against their target fungi at levels below 1 ppm. On that basis, the dilution effect of the soil would probably preclude the use of soil-incorporated products. In practice the situation is much worse because of the difficulty in achieving complete ground cover and presentation of the product in the infection court.

Beyond that, the physicochemical characteristics necessary for a fungicide to act via the soil are well understood. The demand is for highly active compounds with moderately low lipophilicity, to avoid adsorption to soil particles and allow redistribution in the soil water, combined with the persistence characteristics that would establish long-term control. For highly mobile compounds, slow-release formulations would provide a means to deliver long-term control. However, the technical targets for persistence and movement are in direct conflict with the registration requirements that govern the use of agrochemicals in soils, effectively removing the development of soil fungicides as an option for take-all control.

Seed treatments

Seed treatments provide the most reliable control; in the USA and Europe the use of triazoles (triadimenol, flutriafol) is known to deliver some protection to roots until early spring. In this case, slow-release formulations would help to provide long-term control.

Continued

Case Study 1. Continued.

Foliar fungicides

Although there is an increasing understanding of the physicochemical parameters that govern fungicide movement in the phloem, there are few fungicide products that can be demonstrated to act in that manner, none of which is active against take-all of wheat. It is likely that until clear technical advances in fungicide delivery and performance are made, the control of take-all will remain a debatable commercial target. However, future developments in the control of this and other soil-borne diseases may focus more on the use of crop biotechnology rather than on the discovery of conventional fungicides.

Table 4.4. Characteristics of different types of fungicide screen.

Type of screen	Amount of test chemical needed	Indicative number of chemicals that can be tested per annum
High-throughput tests	Less than a microgram	100,000
In vitro tests	A few micrograms	10,000
In planta tests		
Detached leaf tests	A few milligrams	1,000
Glasshouse, whole plant sprays	A few grams	100
Outdoor plot trials	A few grams	100

In planta *screens*

In planta screens are the most time-consuming and expensive but also the most predictive of final success. An *in planta* test is one where the pathogen undergoes its full life cycle on 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. 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. This 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. 4.1).

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.

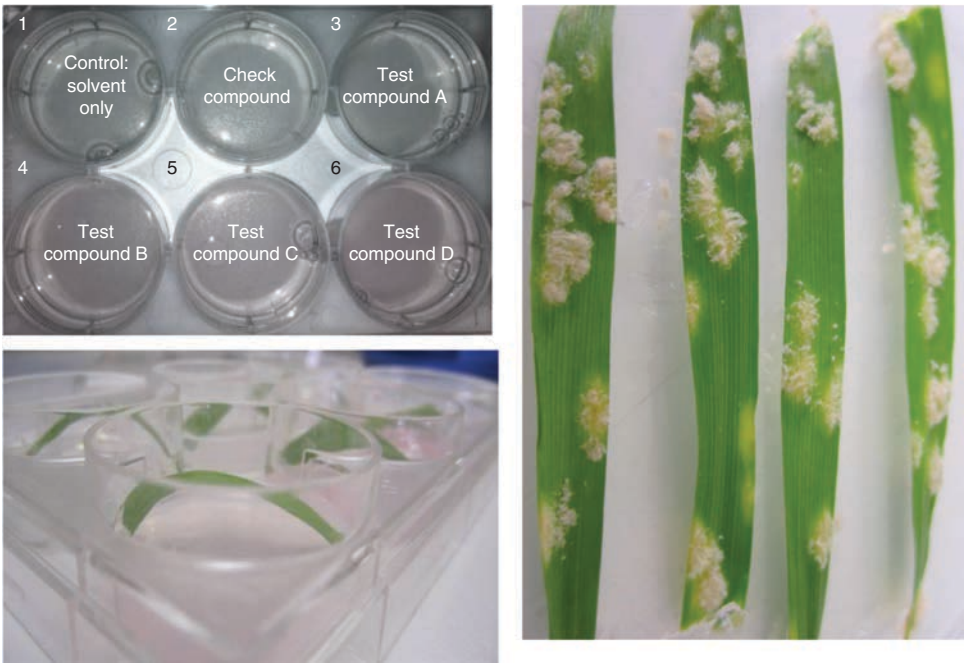


Fig. 4.1. *In planta* test of compounds against ERYSGH. Leaves of a susceptible barley cultivar are excised and placed on an agar suspension containing supplements that inhibit senescence. Each well contains a different compound, but with the same solvent: well 1 has no compound and is a positive control; 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).

In planta screens have the advantage that they tell the researcher whether the compound 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.

In vivo *screens*

In the fungicide industry, *in vivo* refers to the growth of a fungus away from a plant. It is a conceptually simple matter to grow a fungus in an agar plate or microtitre plate-well and to add aliquots of test compounds. *In vivo* tests use much less compound than *in planta* tests.

If the fungus is inoculated into the centre of an agar plate containing the compound, the reduction in radial growth rates 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. Agar plates are large and unwieldy, so companies prefer to use microtitre plates that have 96 wells in an 8 × 12 array. The growth of the fungus can be measured by assaying 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.

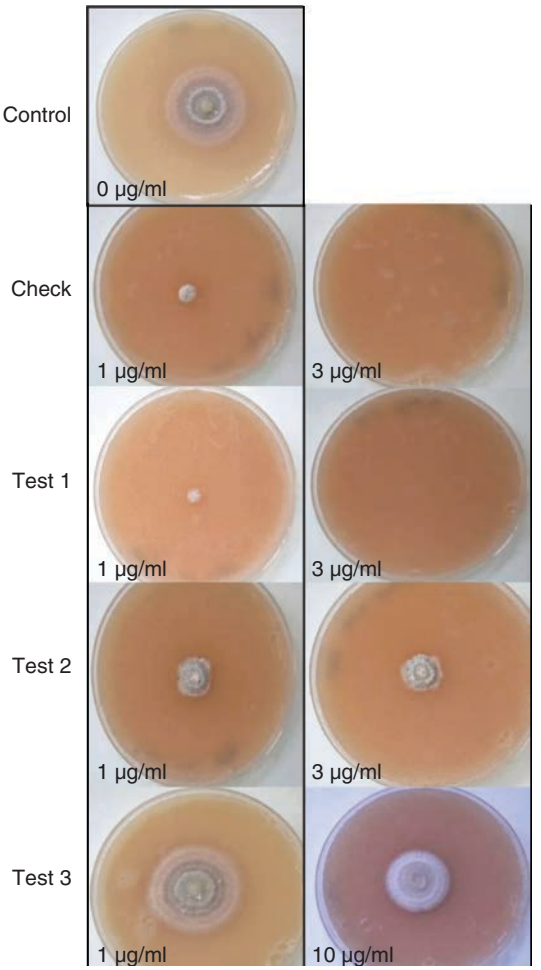


Fig. 4.2. Radial growth assays of LEPTNO. Each plate contains a nutrient agar medium amended at two concentrations with solvent (control), check (current fungicide) or test compounds. The plates are inoculated with spores or a mycelial plug in the centre and allowed to grow for 2–7 days. The average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

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 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.

High-throughput tests

The term 'high-throughput' reflects the frustration of the fungicide industry with the slow pace of fungicide discovery even when using *in vivo* tests. New methods of generating test compounds, such as combinatorial chemistry, led to a backlog of untested

compounds. There was perceived to be a need to develop faster tests. So high-throughput tests encompass a range of tests with the common factor of being faster than an *in vivo* test. The goal was to screen very large numbers of compounds with an assay designed to reflect some essential function of the pathogen. Examples would be an enzyme assay or a bacterial strain with a reporter gene. 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.

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.

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 as 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 taken into account 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 SEPTRI and BOTCIN are favoured for that reason over VENTIN and MYCFIJ. It is, however, an unfortunate fact that many of the priority targets are obligate pathogens; rusts and mildew, both powdery and downy. Furthermore, history shows that obligate pathogens tend to be sensitive to a greater range of fungicides than the facultative pathogens. Hence companies that screened only facultative pathogens would risk missing out on a lucrative mildewcide. An example would be quinoxyfen.

Table 4.5. Characteristics of major fungicide test organisms.

Code/pathogen name	Disease	Host	Taxonomy	Facultative/ obligate
SEPTRI	Septoria tritici blotch	Wheat	Ascomycete	Facultative
PYRIOR	Blast	Rice	Ascomycete	Facultative
UNCNEC	Powdery mildew	Grapevine	Ascomycete	Obligate
ERYSGT/H	Powdery mildew	Wheat and barley	Ascomycete	Obligate
PUCCRT	Brown rust	Wheat	Basidiomyecte	Obligate
PHYTIN	Late blight	Potato/tomato	Oomycete	Facultative
BOTCIN	Botrytis grey mould	Many but especially grape	Ascomycete	Facultative
PLASVIT	Downy mildew	Vine	Oomycete	Obligate
	Phakospora pachyrhiza	Soybean	Basidiomycete	Obligate
VENTIN	Scab	Apple	Ascomycete	Facultative (but very slow growing)
MYCFIJ	Black sigatoka	Banana	Ascomycete	Facultative (also slow growing)

In addition, some non-pathogenic fungi are widely used in fungicide discovery laboratories. These include the yeast *Saccharomyces cerevisiae* and the filamentous species *Aspergillus nidulans* or *Aspergillus niger*. The use of non-pathogenic species is an obvious consequence of the fact that without very few exceptions, all fungicides that have reached commercial release inhibit the growth of at least some fungi in culture. Furthermore, many fungicides are more potent on plates than on plants and hence are more sensitive for lead detection. The non-pathogenic fungi have been used in fundamental science as model systems. Such model systems were chosen because of their ease of culture and fast life cycles. Generations of fundamental scientists have generated extensive genetic resources such as complete mutant libraries and functional genetic technologies. The first fungal genome sequences to be made publically 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 yeasts strains.

Sources of fungicide leads

Screening identifies potential products but, more crucially, the lead compounds from which products are developed. The term ‘lead’ is used widely in the industry. It refers to the first compound that shows activity against a target fungus. The chemical structure is then determined and many variants are synthesized. These variants are also tested in the assays until the structural features associated with activity are identified.

Nearly all current products have arisen from the development of leads. The performance of the end product is likely to be considerably different from that of the early lead compound. What constitutes a lead is subject to continual debate, not least because the commitment of resources to lead development can be critical, and one of two philosophies may be applied.

One approach argues that screens should be extensive and that companies should develop any compound with activity, regardless of its initial commercial potential. Thus a chemical with excellent *in vitro* activity against *A. niger* may be investigated further in the hope that the spectrum can be modified to include more important crop pathogens or that it possesses a novel and useful MOA. To some extent, all companies employ this approach, as all new compounds are submitted to be screened as herbicides, insecticides and fungicides and there are many examples of the discovery of activity in one discipline leading to a product in another.

The first tests within the screen proper may be designed to identify or confirm suspected general activity, or may be focused on those attributes that market surveys define as valuable. For example, the inclusion of fungi drawn from as wide a taxonomic range as possible is the most effective method of exploring efficacy. Value is placed on spectrum rather than on commercial targets, and important pathogens such as obligates may be excluded in favour of more easily managed organisms. *In vitro* techniques enable many fungi to be employed, and because the complications inherent in using infected plants are avoided, it is possible to explore the direct effects of compounds upon fungal development. Further, the use of broad-spectrum tests ensures that the company establishes an excellent historic database that can be interrogated to find suitable leads should new commercial targets be found.

The second philosophy demands that only those pathogens identified as commercially useful are used in screening. While this approach has the clear advantage over a non-targeted system that active leads are more likely to produce valuable products, the comparatively narrow spectrum reduces its utility as a historic database. Also if targets change, the screening test must be modified, resulting in the discontinuity of records. Targeted tests are generally carried out *in vivo*, which changes the balance of resources required from the laboratory to glasshouse and controlled-environment facilities. It also means that the fundamental activity of the candidate fungicides may be masked by physicochemical interactions with the environment surrounding the host plant and residing within the host plant.

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. 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. A recent success for the natural product route is the strobilurins (see Chapter 5). The original set of compounds was extracted from the fungus *Strobilurus tenacellus* (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 improbable partnership of the traditional random approach to pesticide discovery and the novel techniques of combinatorial chemistry was 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, 1994). 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 which can then be screened and, by a series of elimination studies, the active moieties can be defined and re-synthesized 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 a number of 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 4.1 for an explanation of chemical nomenclature rules).

In contrast, the relationship of some chemistry to biological activity (structure–activity relationship) is extremely narrow. Tricyclazole, the active component of Beam, a DowElanco product for use against PYRIOR, is the only member of that chemical series found to have significant activity against the target pathogen. In that case, the discovery was made purely by chance.

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.

Table 4.6. The triazole family of fungicides.

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	Du Pont
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

Box 4.1. 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 active ingredient name, the formal IUPAC (International Union for Pure and Applied Chemistry) name for the active ingredient, the chemical class (often several levels) and the MOA class. The different names are due in part to the 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 *Ascomycota*.

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 of the rules are followed and exceptions are shown below in italics. Furthermore, some linking letters are omitted to improve pronunciation.

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

	Fully unsaturated compounds		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	

Continued

Box 4.1. Continued.

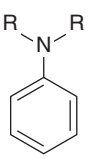
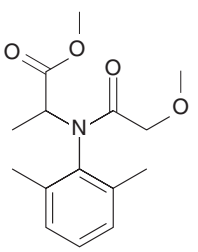
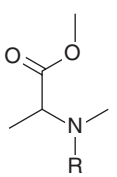
Ring size	Hetero atom	Saturated	FRAC class(es)	Unsaturated	FRAC class(es)
3	N	Aziridine		Azirine	
	N + N			Diazirine	
	N + O	Oxaziridine			
	O + O	Dioxirane			
4	N	Azetidine		Azete	
	O	Oxetane		Oxete	
	N + N	Diazetidine			
	O + O	Dioxetane		Dioxete	
	S + S	Dithietane		Dithiete	
5	N	*Pyrrolidine*		*Pyrrole*	
	O	*Tetrahydrofuran*		*Furan*	C2
	N + N	*Imidazolidine* or *pyrazolidine*		*Imidazole*	G1
	N + N + N			Triazole	G1
6	N	*Piperidine*	G2	Pyridine	G1
	O			Pyran	
	N + N	*Piperazine*	G1	Diazines; *pyrimidine*	A2; G1
	N + O	*Morpholine*	G2	Oxazines	C3
	N + S			Oxathiin	C2
	N + N + N			Triazine	M8

FRAC, Fungicide Resistance Action Committee.

Fused and multiple rings

Many fungicides have fused or multiple rings and an unambiguous systematic naming system would have to be very cumbersome. Instead chemists have tended to focus on natural products and use trivial names.

Irregular pharmacophore classes of the major fungicides are tabulated below.

FRAC class(es)	Chemical group	Example fungicide
A1; RNA polymerase 1	Phenylamide 	Metalaxyl 
	Acylalanine 	

Continued

Box 4.1. Continued.

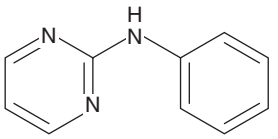
A2; adenosine deaminase	Hydroxy- (2-amino-) pyrimidine	Bupirimate
B1; β-tubulin assembly in mitosis	Benzimidazole	Carbendazim
C2; inhibition of complex II; succinate dehydrogenase	Carboxamide (note: carboxamides are also in C1, C7, I2, P3)	Boscalid
C3; inhibition of complex III; quinone outside inhibitors (QoI)	Methoxyacrylate, etc.	Azoxystrobin

Continued

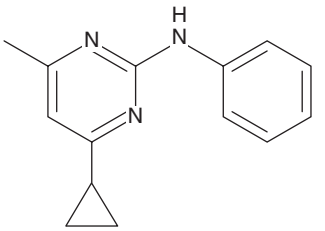
Box 4.1. Continued.

D1; methionine biosynthesis

Anilinopyrimidines

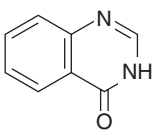


Cyprodinil

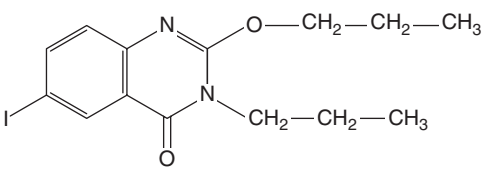


E1; signal transduction (mechanism unknown)

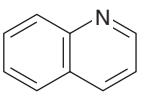
Quinazolinone



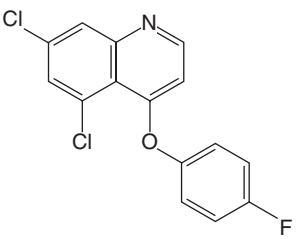
Proquinazid



Quinoline

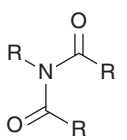


Quinoxyfen

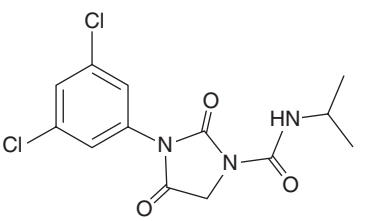


E3; osmotic signal transduction

Dicarboximides

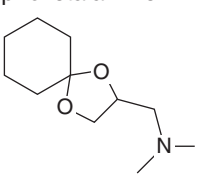


Iprodione

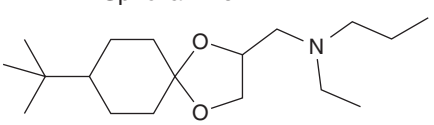


G2; Δ^{14}-reductase and $\Delta^{8}\rightarrow\Delta^{7}$-isomerase in sterol biosynthesis

Spiroketalamine



Spiroxamine



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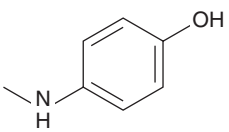
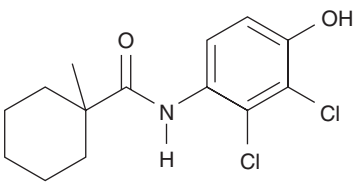
Box 4.1. Continued.

G3; 3-keto-reductase in C4-demethylation (*erg27*)

Hydroxyanilide

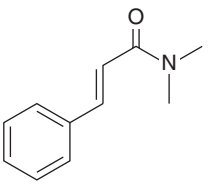


Fenhexamid

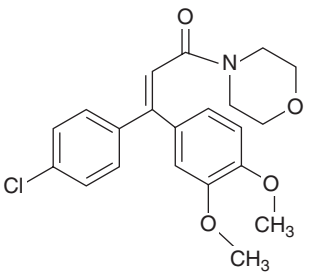


H5; cellulose synthase

Cinnamic acid amides

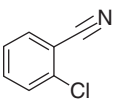


Dimethomorph

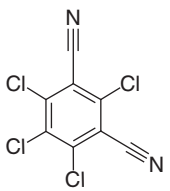


M5; multi-site chloronitriles

Chloronitrile

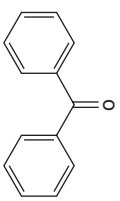


Chlorothalonil

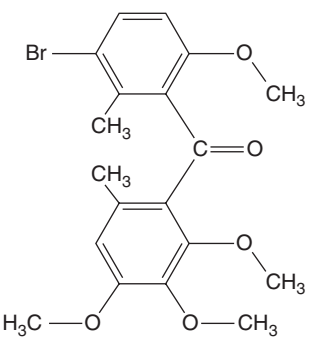


U8; unknowns

Benzophenone



Metrafenone



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 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 (3D) 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 cell-free assays, may lack *in vitro* or, more commonly, *in vivo* 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 3D 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 has been modelled (Fig. 4.3). 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).

Many attempts to design novel chemistry to fit known sites of action have failed. An illustration is given in Case Study 2.

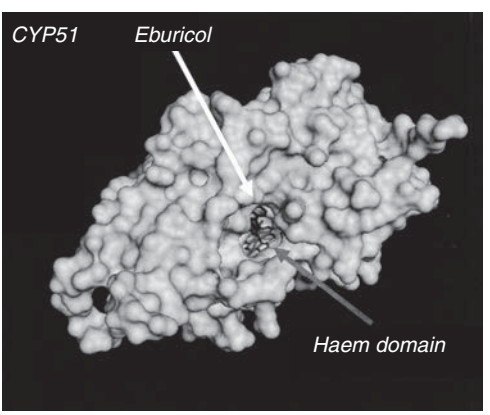


Fig. 4.3. Three-dimensional structure of fungal CYP51 showing the haem active group and the binding site of the substrate eburicol. Such structure allows the *in silico* docking of compounds to predict inhibitory activity prior to the decision whether to synthesize.

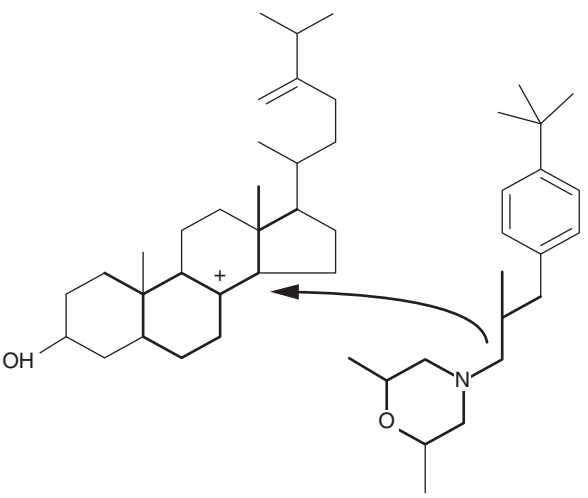
Case Study 2. Carbocation mimicry in amidiniums and guanidiniums.

Morpholine and piperidine fungicides inhibit sterol biosynthesis at the Δ^{14}-reductase and $\Delta^{8}\rightarrow\Delta^{7}$-isomerase enzyme steps, through their action as transition-state analogues of the natural sterol substrate. Using structural mechanisms that stabilize the transition state, they bind strongly to the enzyme and reduce the activation energy of the enzymic reaction.

In sterol biosynthesis, reduction of the Δ^{14} double bond and isomerization of the Δ^{8} double bond are probably mediated by a chemical intermediate known as a carbocation. The protonation of fenpropimorph and piperidine, which occurs at physiological pH, results in the formation of similar intermediates and may explain their activity against powdery mildews, especially ERYSGH. This mechanism was examined by Liebeschuetz and co-workers (Arnold *et al.*, 1995) at DowElanco as a likely target for a directed synthesis programme and work began on the rational design of carbocation mimics. A binding model for fenpropimorph was adopted as a guide for synthesis (Fig. 4.4).

The chemical starting points for the synthesis programme included guanidinium and amidinium structures (Fig. 4.5). The lead compounds conformed with the theoretical fit to the $\Delta^{8}\rightarrow\Delta^{7}$-isomerase and Δ^{14}-reductase carbocationic intermediates (Fig. 4.6). Both compounds were confirmed as active in screening tests against ERYSGH and PUCCRT, at levels equivalent to fenpropimorph. In cell-free enzyme assays derived from *Ustilago maydis*, the lead compounds had activity at the micromolar level which tended to favour interest in the amidinium salt (IC_{50} guanidinium = 30 μM; IC_{50} amidinium = 20 μM). However, in concurrent tests fenpropimorph was superior with an IC_{50} = 0.35 μM.

Subsequent modifications of the lead compounds concentrated in three areas (Fig. 4.7), producing a guanidinium series of 11 compounds and an amidinium series of nine compounds. There was a good correlation between *in vivo* and cell-free assay results for all compounds, but in whole-cell assays the initial activity of the lead compounds and their analogues was drastically reduced, in contrast to the maintenance of high



Fenpropimorph

Fig. 4.4. Binding of fenpropimorph to the isomerase carbocationic intermediate.

Continued

Case Study 2. Continued.

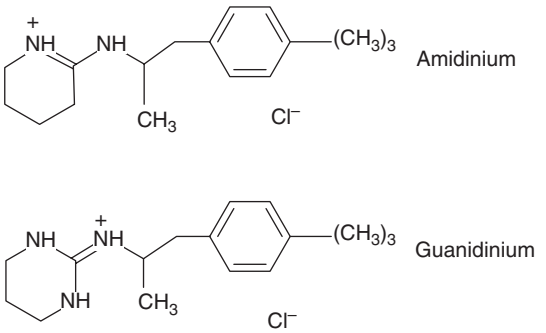


Fig. 4.5. Lead compounds used in the synthesis programme of piperidine and morpholine fungicides.

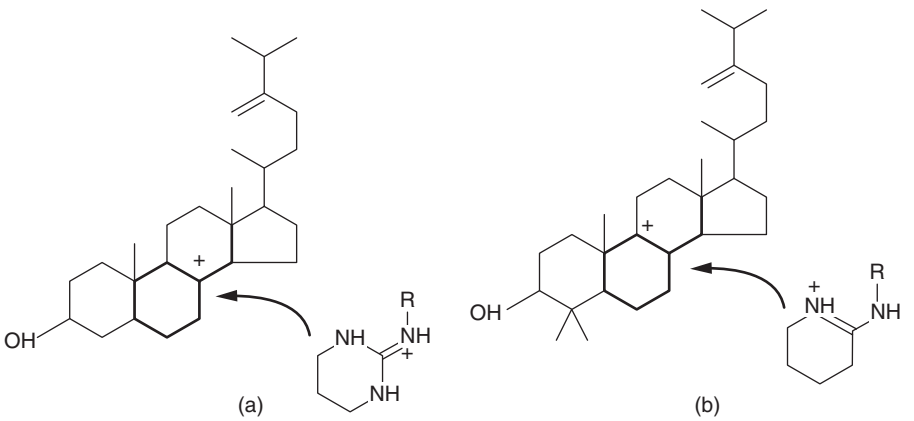


Fig. 4.6. Proposed fit of the cyclic guanidinium (a) and amidinium (b) over the $\Delta^8 \rightarrow \Delta^7$-isomerase and Δ^{14}-reductase carbocationic intermediates.

levels of inhibition by fenpropimorph. Subsequent *in vivo* tests carried out using commercially acceptable application techniques and a spray volume of 300 l/ha demonstrated that neither the guanidinium nor the amidinium series of compounds controlled powdery mildew as effectively as the standard, fenpropimorph. The disappointing levels of disease control were attributed to a lack of redistribution within the crop and a lack of uptake into the target pathogen.

The ability to redistribute is a crucial factor in the success of cereal fungicides. Application volumes high enough to wet leaves or to cover them extensively are used routinely in broad-leaf crops such as grapevine and top fruit in which a component of disease control is the extensive use of immobile surface protectants. These require good coverage in order to operate effectively and volumes as high as 1000 l/ha are not uncommon. In cereals, the major part of disease management is through the use of systemics or compounds such as fenpropimorph that operate in part through the vapour phase. These are less reliant on application volume and treatments are made in spray-tank solution

Continued

Case Study 2. Continued.

(250–300 l/ha). Acceptable efficacy levels of the exploratory guanidiniums and amidiniums appeared to be restricted to high-volume systems, suggesting a mobility problem.

It was also concluded that in whole-cell and *in vivo* tests the barriers to penetration of the polar and highly basic test compounds (pK_a = 10–12) prevented the expression of their intrinsic activity against the target enzymes. Fenpropimorph, however, has a much lower acid strength (pK_a = 7) and *in vivo* is able to cross membranes in an un-ionized form.

The research demonstrates the importance of a holistic approach to discovery which relates biochemical activity to practical performance in a multi-disciplinary fashion. It also clearly shows the advantages of an effective and directed approach to the synthesis of potent inhibitors.

Chemorational design

A further input to the development of a lead is to modify it in ways that are designed to optimize the physicochemical properties of the compound (Fig. 4.7). This process is part science and part art. Chemists use a multitude of inputs to decide how to modify compounds to improve their activity, increase their stability, decrease their toxicity and decrease the costs of synthesis. Also, they must bear in mind the patent situation and seek to make compounds that bypass competitors' patents.

More than 200 compounds have been commercialized as fungicides and many thousands have failed to progress, so there is a good deal of experience of the types of physicochemical properties that are compatible with good fungicidal field performance. Chemists focus on the melting and boiling points and the vapour pressure as these reflect the degree to which the compound will vaporize after application on

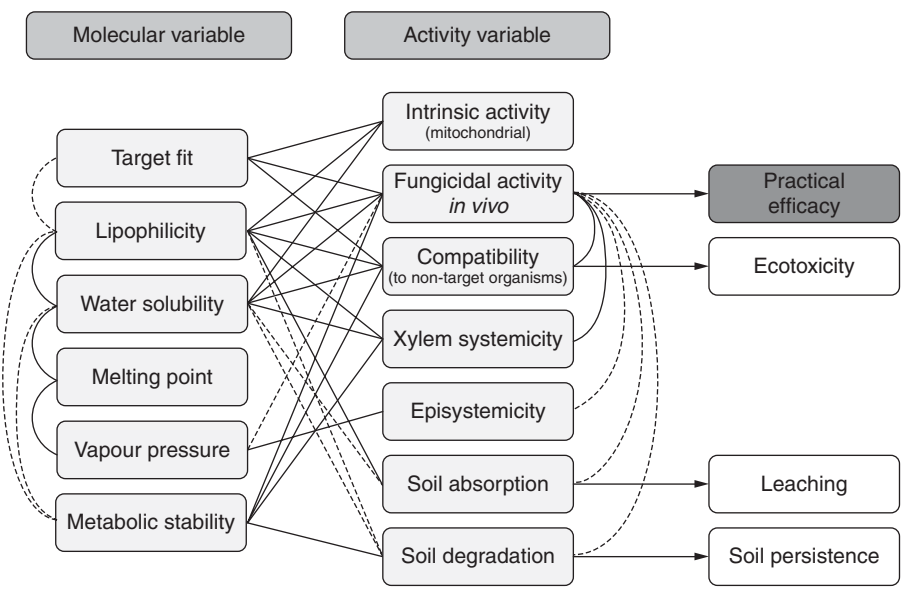


Fig. 4.7. Structure–activity relationships: the complex network between variables. (From Krämer *et al.*, 2012.)

the leaf. They also focus on the log *P*, which is the logarithm of the partition coefficient between n-octanol and water. This parameter measures hydrophobicity/hydrophilicity and predicts whether the compound will be mobile in the leaf and how it will be formulated. They also focus on molecular weight as large compounds will be more unstable. Most fungicides have a molecular mass between 100 and 300 Da.

Screening methodology

Target-orientated screening concentrates resources on those opportunities that are commercially viable and minimizes the risk of developing compounds that will never provide sufficient return to justify the investment in discovery. The testing cascade which forms the screen includes the following activity and performance determinants:

- *Activity* – target pathogens and their hosts.
- *Performance* – persistence, application timing and method, mobility and resistance management.

Company strategy is reflected in the composition of the discovery screen. If the biological targets carry equal commercial merit, the screen may be broad-based in design and include all pathogens and their hosts at the first or primary test level. Subsequent testing then concentrates on the demonstrated efficacy spectrum. Because of the high rates normally employed at the primary level, many submitted compounds demonstrate some fungicidal activity and are elevated to more stringent, secondary examination. This is usually a rate–response study to determine the rate at which the test compound loses efficacy, compared with a suitable standard fungicide. Further tests in the final stages of laboratory and glasshouse screening begin to define the influence of performance attributes on biological efficacy. A screen of this type uses a process of elimination to discover candidate fungicides and can be likened to a series of sieves, with the coarsest sieve being the first test.

However, where resources are limited, the system may be rationalized to include only those targets that are deemed essential, either as commercial targets or as models on which to base further work. Subsequent testing is always directed towards the evaluation of commercially important attributes, defined by marketing and by the financial return required by the individual company. A screen of this type has a comparatively narrow primary level base but then expands, based upon initial activity, to include taxonomically related targets and performance attributes before focusing on the most active and/or commercially acceptable candidate. Given that user, consumer and environmental safety are absolute requirements and subjects for later study, screening concentrates on the definition of those characteristics that will make an effective, reliable and flexible product. These are usually arranged in the screen in order of decreasing priority and increasing complexity.

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 (see Case Study 3).

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

Case Study 3. A rationalized fungicide screen.

This case study describes an extreme but not unrealistic scenario which is resource-limited and driven entirely by primary targets that individually represent markets of sufficient size to support a product. Thus ERYSGT, PYRIOR, PLASVIT and SEPTRI alone merit inclusion. Activity against any one triggers a cascade of tests designed to establish a possible commercial fit with a marketing objective (Table 4.7).

Specific activity against any single powdery mildew is uncommon and so ERYSGT serves as a general model for activity against powdery mildew fungi. It also activates tests against secondary pathogens which together with ERYSGT form part of a commercial target. Thus, tests against PUCCRT and *Pseudocercosporella herpotrichoides* follow successful primary level activity against ERYSGT. Similarly, VENTIN is included at the secondary level as in top fruit the target market is for a combined apple scab and powdery mildew fungicide.

In grapevine, PLASVIT represents both a commercial target and a model for oomycete fungicides. Secondary tests with compounds showing primary level activity against PLASVIT trigger tests against PHYTIN, a pathogen of secondary commercial importance.

Tests against *R. solani* in rice are prompted by activity against PUCCRT (both are basidiomycetes) and by good control of PYRIOR, the major target for rice fungicides.

At the tertiary level, activity against SEPTRI triggers studies against MYCFIJ, black sigatoka disease of banana.

Table 4.7. Rationalized fungicide screening cascade.

Primary level	Secondary level	Tertiary level a	Tertiary level b
ERYSGT	ERYSGT	ERYSGT	ERYSGT
	UNCNEC	UNCNEC	UNCNEC
	Apple powdery mildew	Apple powdery mildew	Apple powdery mildew
	VENTIN	VENTIN	VENTIN
	PUCCRT	PUCCRT	PUCCRT
	Eyespot	Eyespot	Eyespot
			Barley net blotch
			Barley scald
			Barley leaf rust
			Wheat yellow rust
SEPTRI	SEPTRI	SEPTRI	SEPTRI
	LEPTNO	LEPTNO	LEPTNO
	PUCCRT	PUCCRT	PUCCRT
	Eyespot	Eyespot	Eyespot
			MYCFIJ
			Barley leaf rust
PLASVIT	PLASVIT	PLASVIT	PLASVIT
		PHYTIN	PHYTIN
			Pythium
PYRIOR	PYRIOR	PYRIOR	PYRIOR
		Rhizoctonia solani	*Rhizoctonia solani*

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.

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 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.

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

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 active ingredient in a practical form.

Formulated products contain the active component alone or in combination with other actives in a stable form under a wide range of environmental conditions. They should be straightforward to use and should deliver the fungicide in a manner that maintains its intrinsic activity or increases its performance through enhanced redistribution or mobility. In some cases, inventive formulation may enhance 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.

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. Conversely, losses of product must be minimized. Similarly, surface-acting fungicides may be held on the leaf in a variety of ways, but formulation components that (for example) prevent wash-off in rainstorms by increasing uptake of the fungicide into the plant remove the active material from the site of disease control. The instability of the strobilurin analogue kresoxim-methyl in plants has established the need to minimize penetration (Gold *et al.*, 1994).

The addition of adjuvants can profoundly affect the performance of fungicides and they are routinely screened in combination with new materials. 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). 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. 4.8; Stock, 1996). Such modifications may be advantageous or disadvantageous depending upon the proposed treatment timing and the growth pattern of the target pathogen. In some cases, formulation may inhibit fungicide action, as in the removal of activity of prochloraz in wettable powder formulations. Fungicides are formulated in several ways, depending on their physical characteristics and on the needs of the market.

Wettable powders are solid formulations suitable for compounds that have low aqueous solubility. They 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 (ultraviolet filters). Wettable powders are by their nature dusty and are potentially hazardous to handle. However, many immobile fungicides are formulated as wettable powders.

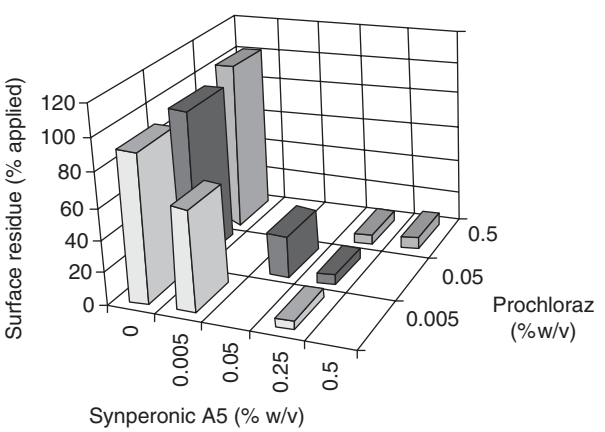


Fig. 4.8. Effect of Synperonic A5 on uptake of prochloraz into wheat leaves. (From Stock, 1996.)

Dust formulations are similar to wettable powders in that they 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 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.

Granule formulations are produced by the adsorption of fungicide on to the surface of porous clay pellets, ranging from 0.5 to 1.5 mm in diameter. A modification driven by new spray technology is the microgranule (100–300 μm diameter) that is designed for use in spinning disc applicators. Granule formulations are easy to apply, are stable in high wind and, being a relatively heavy solid, have good crop penetration characteristics. Granules are frequently used in rice paddies where quick and effective applications can be made by hand.

Suspension concentrates 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 plus suitable adjuvants. 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.

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 rapidly separate 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.

Emulsifying agents can be anionic, cationic or non-ionic. Non-ionic agents, for example polyethylene ethers, improve fungicide coverage on the often waxy surfaces

of target crops by reducing 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.

Because of their toxicity and fire hazard, organic solvents are being replaced by alternatives; for example, microemulsions. Where the active fungicide is soluble in water, the material may be formulated as a water-miscible liquid.

Application

Fungicides may be applied to crops as seed treatments, in foliar sprays or smokes or as fruit dips. Most application methods are universally used for all pesticides and an overview of only the major types is presented.

Seed treatments

Fungicide seed treatments are common. Fungicides designed to be used as seed treatments are of increasing importance. For example, all commercially important cereal seed is treated. Seed treatments (as distinct from seed dressing which refers to a cleaning process, as in the removal of lint from cotton seed) include adhesive dusts, the use of slurries and solutions applied as sprays to seed batches or by immersion. Historically, the use of seed treatments was confined to immobile fungicides such as the organomercurials, but they are now employed routinely to apply systemic materials in a convenient and economic manner. There is considerable interest in the use of slow-release seed treatments of systemics to provide long-term control of crop disease.

Foliar treatments

Most fungicides are diluted in water before application. The mixture is delivered through atomizing nozzles operating under high pressure and designed to disperse fine droplets of the product evenly throughout the crop. Volumes of application vary according to the crop and the activity of the product. Traditionally, immobile protectants are applied in high volumes (>600 l/ha) to ensure good coverage. However, the performance of systemics is less affected by poor coverage and they are applied at lower volumes (100–250 l/ha). There is an increasing trend towards a reduction in volume rates (<100 l/ha) through the use of air-assisted sprayers and higher-ground-speed vehicles. This applies especially to areas like Australia where water is at a premium.

Fungicides used in fruit are a mixture of immobile protectants and systemics, and programmed or repeat spraying is required to achieve acceptable disease control. Spray volumes in fruit tend to be high. In cereals, most compounds are systemic or are redistributed via the vapour phase. Spray frequency is lower than in fruit and application volumes tend to be in the 200–300 l/ha range. Handheld or tractor-mounted

spraying is difficult in some crops and aerial applications are used. In bananas, for example, black sigatoka disease is controlled by the programmed use of fungicides applied in ultra-low volume. In this technique, which uses volumes of about 20 l/ha, oil rather than water is used as the diluent.

Droplet size, nozzle type, operating pressure and formulation are interdependent variables in the application of fungicides. Tractor-mounted, conventional spraying produces a wide range of droplet sizes and can result in the loss of product via drift or due to low retention on the target leaf surfaces. The optimization of droplet size ensures more effective plant coverage, and several systems have been introduced. Spinning disc applicators rely on the delivery of the fungicide spray solution on to a rotating disc. The speed of delivery to the disc, the rotational speed of the disc and its diameter control droplet size. A further development of this technique is electrodynamic spraying. In this technique, a positive charge is imparted to the fine droplets as they leave the surface of the disc. The particles are attracted to the negatively charged crop and little spray is lost. In high-density plantings or when the target pathogen lies deep within the crop canopy, electrodynamic spraying fails to deliver the fungicide in an acceptable manner, most of the product being retained by the upper leaves. In practice, neither of the systems based on spinning discs has found acceptance within the farming community other than in small areas of crops or in protected crops.

Fungicides may also be applied in smokes, where the active ingredient is delivered during burning of the formulated product. This technique is commonly used in glasshouses.

Applications of fungicides in granular form direct to the roots are used in glasshouses, but have a major application in rice nursery beds where they provide the farmer with a convenient and effective method to deliver rice blast and sheath blight fungicides.

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