6 Fungicide Resistance

Key Points

- Fungicide resistance is a critical factor in the development and use of fungicides.
- Resistance affects the majority of current major fungicide classes.
- The study of fungicide resistance has been impacted significantly by genomics.
- Fungicide resistance can be managed by careful use of integrated disease management principles and by using minimum doses, mixtures and alternations of fungicides.

Introduction

Resistance to fungicides has grown in importance in the last 20 years and now ranks as the central preoccupation of the fungicide industry. Despite extensive fungicide use in the previous 90 years, resistance emerged as a practical problem as recently as 1970. Significantly, the incidence of resistance has been restricted largely to systemic fungicides that operate against single biochemical targets (single-site inhibitors). These were introduced from the mid-1960s onwards and include the majority of the major newer groups of fungicides (Table 6.1).

Resistance to fungicides is manifested as failures of previously efficacious products to control disease. In such circumstances, the entire economic rationale of fungicide use is removed. 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, and also farmers and consumers, that the efficacy of fungicides is protected as far and for as long a period as is possible. Hence the industry has united to form the FRAC (www.frac.info) which collates information and dispenses advice.

Crop losses resulting from a breakdown in disease control can be spectacular, as occurred in northern Greece following the outbreak of benzimidazole resistance of *Cercospora beticola* in sugarbeet and in Western Europe following the loss of metalaxyl control of PHYTIN. The consequent crop husbandry and financial implications were significant, involving changes in management practice and potential yield loss (Pasquereau, 1994).

More recently, fungicide resistance was observed in barley powdery mildew populations grown in Western Australia. Very susceptible cultivars had been grown for 10–20 years. When disease problems emerged, cheap triazole fungicides, especially tebuconazole, were widely and exclusively used. As a result mutant strains of ERYSGH emerged. The resulting losses were estimated at AUS$100 million per annum or about

Table 6.1. Major instances of fungicide resistance. (From http://www.frac.info/.)

Group name (abbreviation)	Fungicide common name(s) (a selection)	Risk level; high or medium or low (current assessment)[a]	Years between introduction and emergence of field resistance[b]	Comments
A1; Phenylamides (PAs)	Benalaxyl Metalaxyl	H	2	Cross-resistance in various oomycetes
A2; Hydroxy-(2-amino-) pyrimidines	Bupirimate Ethirimol	M	2	Cross-resistance in various powdery mildews
B1; Methyl benzimidazole carbamates (MBCs)	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; *N*-Phenylcarbamates	Diethofencarb	H	Not known	Target site mutation in β-tubulin gene: E198K. Negative cross-resistance to MBCs
C2; Succinate dehydrogenase inhibitors (SDHIs)	Carboxin Bixafen Sedaxane Boscalid	M to H	3	Several target site mutations known; cross-resistance observed. Apparent fitness penalty. Medium RFs
C3; Quinone outside inhibitors (QoIs)	Azoxystrobin Picoxystrobin Pyraclostrobin Trifloxystrobin	H	2	Target site mutations G143A and F129L. Cross-resistance. High RFs for G143A. Intron at 143 protects against resistance
D1; Anilinopyrimidines (APs)	Cyprodinil Mepanipyrim Pyrimethanil	M	5	Target site mutations in BOTCIN

E1; Quinolines	Quinoxyfen Proquinazid	M	4	Cross-resistance known. Fitness penalty
E3; Dicarboximides	Chlozolinate Iprodione Procymidone Vinclozolin	M	5	Resistance common. Target site mutation in OS-1 I365S
G1; Demethylation inhibitors (DMIs) (sterol biosynthesis inhibitor (SBI) Class I)	Prochloraz Fluquinconazole Metconazole Propiconazole Tebuconazole Tetraconazole Prothioconazole	M to H	7	Resistance is common with many combinations of mutations in *Cyp51* gene(s), promoter mutations in *Cyp51*. Moderate RFs. Cross-resistance moderate to high within DMIs; variable and sometimes negative with other SBI classes. Also efflux pump mutation especially in BOTCIN
G2; Amines ('morpholines') (SBI Class II)	Fenpropimorph Tridemorph Spiroxamine	L to M	34	Sensitivity shifts observed
G3; (SBI Class III)	Fenhexamid	M	12	Field experiments
H5; Carboxylic acid amides (CAAs)	Dimethomorph Flumorph	H	2	Target site mutations known in *CesA8* genes
I2; Melanin biosynthesis inhibitors–dehydratase (MBI-D)	Carpropamid	M	6	Field resistance known

[a]H, high; M, medium; L, low.
[b]Data from Brent and Hollomon (2007a,b).

AUS$20/ha. Fortunately, the introduction of new fungicides from different MOAs and the replacement of the most susceptible cultivars are expected to reduce the disease to an acceptable level within a 3- to 6-year timeframe (Tucker *et al.*, 2014).

Definitions

The fungicide resistance literature has a confusing vocabulary. As in all areas of science, it is important to be clear what various terms mean.

Resistance and sensitivity

Resistance and sensitivity are different sides of the same coin. A rough test is to grow fungal isolates on a concentration of fungicide that controls wild-type strains. This dose is known as the 'discriminatory dose' (DD; Fig. 6.1). Strains that can grow on the DD are said to be resistant.

A more precise technical 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 or EC_{50}. EC_{10} and EC_{90} (the concentration required to inhibit growth by 10% or 90%) are also used for some purposes. EC_{50} values apply to one strain rather than a species as a whole.

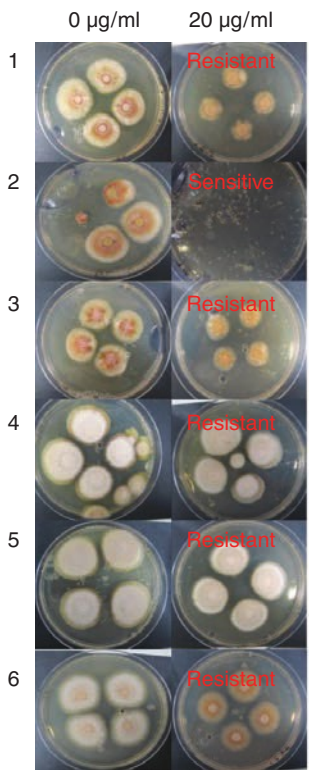


Fig. 6.1. A discriminatory dose test for six strains of *Ascochyta lentis* using thiabendazole at 20 µg/ml for 7 days. Strain 2 was classified as sensitive while the other strains were classified as resistant.

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 carried out before a fungicide is introduced into new regions.

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 has reached 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 (Fig 6.2). Growth of the fungi is measured by turbidometric measurements using a microplate reader. Large amounts of data can be acquired directly to computer. The EC_{50} calculations can be automated and the data stored for future use. Microplates are, however, only suitable for fungi that can be induced to form spores in culture.

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 6.3 illustrates such an assay for ERYSGH and tebuconazole. These assays are the most requiring of time, space and material.

Resistance can be intrinsic or acquired. Intrinsic resistance is a property of the species. Thus oomycete fungi are resistant to triazoles; intrinsic resistance is related to 'spectrum'. Acquired resistance is a property of individual strains within a species.

Resistance factor

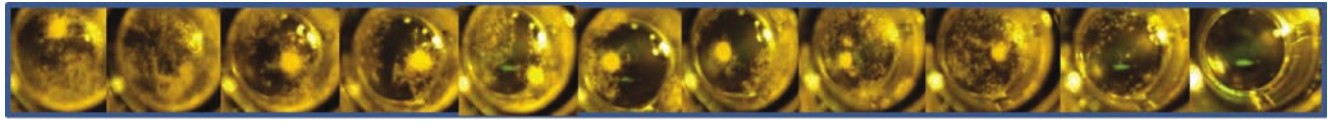
The resistance factor (RF) is the ratio of the EC_{50} of a 'resistant' isolate to that of an apparently normal or sensitive isolate. Isolates of a pathogen vary in myriad properties and so EC_{50} values will vary between isolates of a sensitive or naïve (i.e. one that has not been exposed to the fungicide) population. Such variation can be a factor of ten or 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 (<5), moderate (5–20) and high (>20). Higher RFs occur when the mutation giving the resistance gives a very high level of resistance. In some circumstances, low or moderate RFs are termed tolerance rather than resistance. It can also be called 'lower sensitivity'.

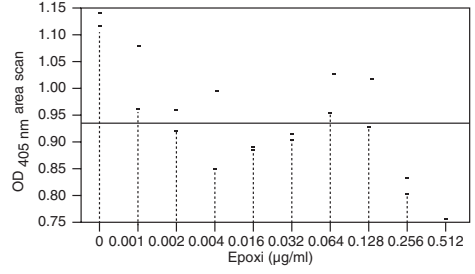
Field resistance

Field resistance is what really matters. It can be defined as the failure of a fungicide applied efficiently at the maximum permitted rate and frequency to give adequate control of the disease. Its occurrence depends on two factors:

(a)



(b)



Level		Mean
0	A	1.1290000
0.001	A B	1.0200000
0.064	A B C	0.9915000
0.128	A B C	0.9720000
0.002	A B C D	0.9410000
0.004	B C D	0.9220000
0.032	B C D	0.9085000
0.016	B C D	0.8875000
0.256	C D	0.8175000
0.512	D	0.7580000

(c)

Epoxi concn (µg/ml)	Log Epoxi concn	% inhibition	Log % inhibition
0	N/A	0	N/A
0.001	−3	0	N/A
0.002	−2.698970004	0	N/A
0.004	−2.397940009	0	N/A
0.016	−1.795880017	0	N/A
0.032	−1.494850022	31.66818044	1.500623111
0.064	−1.193820026	30.42688465	1.483257488
0.128	−0.89279003	45.9582198	1.662363198
0.256	−0.591760035	63.03360581	1.799572151
0.512	−0.290730039	87.28428701	1.940936069
1	0	95.64032698	1.980641052

(d)

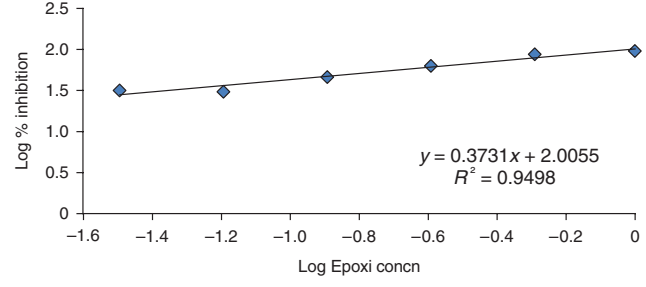


Fig. 6.2. (a) Growth of a non-obligate fungus in a microtitre plate with increasing concentrations of epoxiconazole (Epoxi) for 48 h. (b) One-way analysis of OD_{405nm} area scan versus Epoxi concentration (left) and table showing mean OD_{405nm} of replicate tests (right). (c) Table showing log transformation of Epoxi concentration and of percentage growth inhibition. (d) Plot of log percentage inhibition against log concentration and its use to calculate the EC_{50} of 0.151 µg/ml.

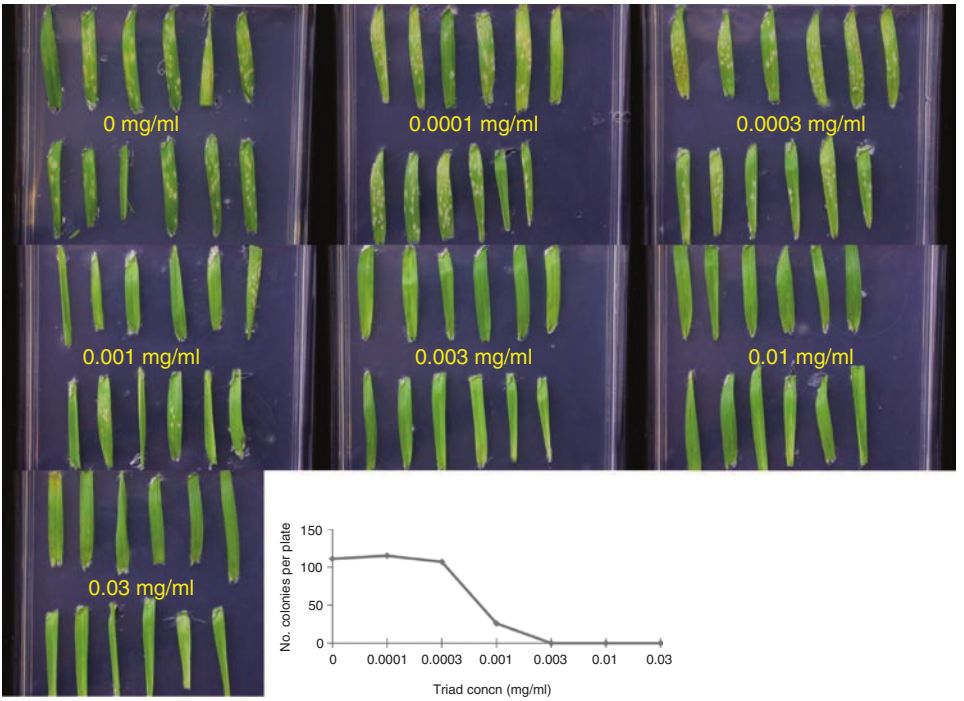


Fig. 6.3. Barley leaves infected with a single spore-derived isolate of barley powdery mildew were placed on benzimidazole agar amended with increasing concentrations of triademifon (Triad). The ED_{50} is estimated to be close to 0.001 mg/ml.

1. Whether the RF of resistant strains is high enough to protect the fungus against the field rate of the fungicide.
2. Whether the prevalence of the resistant strains is high enough to enable them 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 with QoI and MBC fungicides. Partial cross-resistance applies when the RF with one fungicide is much lower than with another. This is the case with triazole fungicides.

Most cases of cross-resistance involve fungicides from the same MOA. Indeed, cross-resistance has often been critical evidence identifying and linking the MOAs of different fungicides as was the case with the CAA fungicides (Blum *et al.*, 2010). Cross-resistance typically involves target site mutations where mutations are found in the gene encoding the target site.

Cross-resistance is normally described as positive; that is, the resistant strain is more resistant to both fungicides than the wild-type strain. Or to put it

another way, both RFs are greater than 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 more tightly than does the wild-type target site. It has been observed in fungicides that target β-tubulin and the *Cyp51* gene.

Multiple resistance

Where cross-resistance involves fungicides from different MOAs, the mode of resistance (MOR) is likely to involve non-target site mutations. These are mainly alterations in efflux pumps. Such pumps are capable of restricting the inflow of fungicides from multiple different classes and thereby decrease the intracellular concentration. Efflux pump resistance has been observed particularly in BOTCIN (Mernke *et al.*, 2011; Leroux and Walker, 2013). Unlike herbicides and insecticides, resistance due to conjugation of the pesticide to glutathione or sugars has not yet been observed in fungi.

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}. 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 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' as the wild-type sensitive population in the *absence* of the fungicide (or in the presence of a fungicide with a different MOA). 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.

There are many potential reasons why a resistant population might carry a fitness penalty. It may be that the target site mutation which confers resistance has the side-effect of reducing the efficiency of the enzyme at the target site. This appears to be the case for *Cyp51* and SDHI fungicides. In the case of efflux pump resistance, it may be that the metabolic energy required to synthesize and drive the pumps represents a significant drain on the 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.

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 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. A number of 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 (Paveley *et al.*, 2014). When diseases are poorly controlled the fungal population size expands rapidly to a number that is large compared with the size of its genome and the number of genes carried. Fungi typically have genome sizes of 40 to 100 million base pairs and express 10,000 to 20,000 genes. Normal processes of spontaneous mutation caused by ultraviolet 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 a billion spores are produced in a pathogen population, most base pairs in the genome would be altered in at least one strain that is present. It has been estimated that 100 m^2 of barley infected with powdery mildew would have a 95% chance of containing a strain with a given mutation (Brent and Hollomon, 2007a,b). 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.

When a mixture of the mutant strain and the wild type has been treated with a fungicide, the normal evolutionary processes come into play. A high proportion of the wild-type strain will be killed by the fungicide whereas some at least of the mutant population (and a higher frequency than the wild type) will survive and reproduce. The proportion of the population that is resistant will increase but it is unlikely to be high enough to be immediately noticeable. However, if the pathogen population reproduces frequently and the fungicide selection 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. The result then is field resistance. Thus pathogen species that reproduce multiple times within a season are higher risk. Or to put it another way, pathogens with short latent periods are high 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. 6.4).

High fecundity is associated with pathogens that produce wind-borne spores primarily. Rain-splashed spores are intermediate in resistance and water-borne and soil pathogens are the lowest risk.

Some cases of fungicide resistance involve mutations in more than one gene. In other cases, the fungicide resistance mutation was in a strain that was only weakly virulent on the crop cultivar used in that field; another strain of the same pathogen had mutated to be strongly virulent on the crop cultivar but had not acquired the fungicide resistance mutation. In both these cases, combinations of genes would be much more

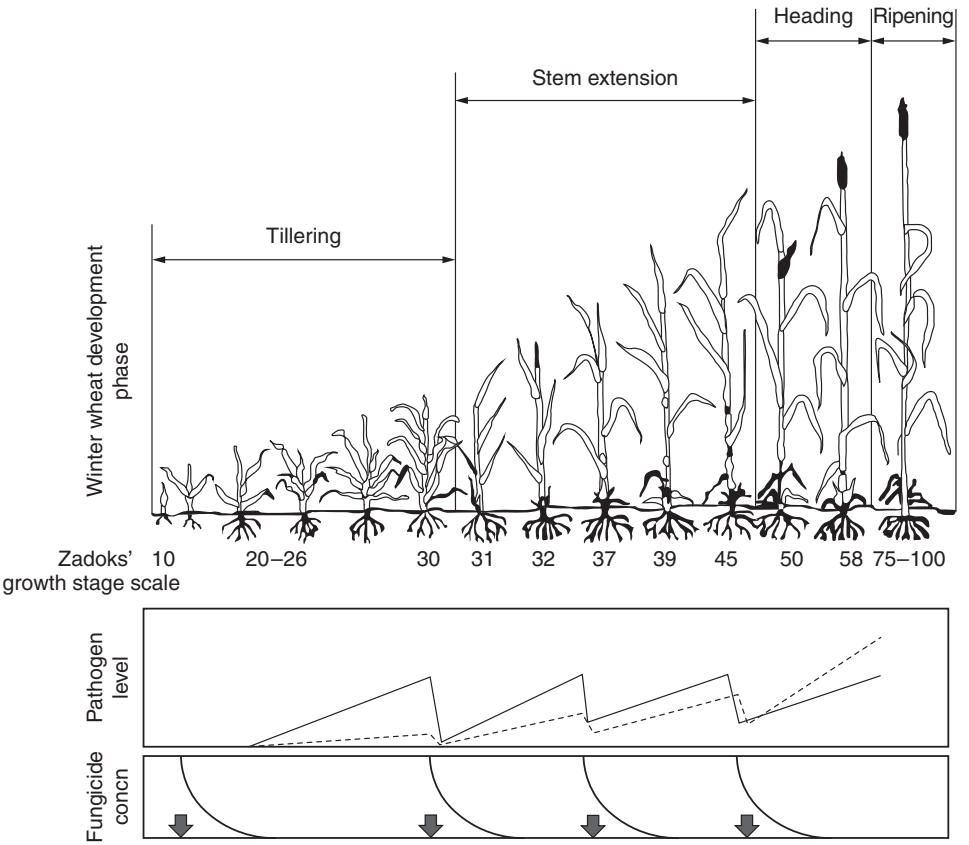


Fig. 6.4. A polycyclic pathogen with a short life cycle controlled by multiple fungicide sprays (fungicide applications arrowed) is at high risk of resistance evolution (– – –, resistant; ——, susceptible).

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.

The asexual or epidemic growth stage of most plant pathogens is haploid. Consequently, mutational changes are expressed immediately and, provided the mutant is fit, its development in the fungal population is rapid. A notable exception is the oomycete fungi in which the asexual stage is diploid and the haploid phase is generated during the sexual stage of development. Similarly, in the *Basidiomycota*, such as the rusts, each cell is a dikaryon (binucleate) and performs as a diploid.

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 6.2 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. BOTCIN, powdery mildews, MYCFIJ, PLASVIT and PHYTIN have consistently been the first species to display resistance to fungicides.

Table 6.2. Fungicide resistance pathogen risk factors.

Pathogen	Fecundity	Latent periods	Sexual reproduction	Resistance prediction	Resistance history
Rhizoctonia	Low	Few	No	Low	Low
Rusts	High	Many	Yes (some)	High	Low
Soil-borne pathogens; smuts and bunts	Low	Few	Some	Low	Low
SEPTRI	Medium	Medium	Yes	Medium	High
Rhizoctonia solani	Medium	Medium	No	Medium	Medium
BOTCIN	High	Many	No	High	High
Powdery mildews	High	Many	Yes	High	High
PYRIOR	High	Many	No	High	Medium
VENTIN	Medium	Medium	Yes	High	High
MYCFIJ	High	Medium	No	Medium	High
PLASVIT	High	Medium	Yes	High	High
PHYTIN	High	Many	Yes (since 1990 in Europe)	High	High

One unexpected exception is the 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. One postulated explanation is the diploid nature of the infective organism. If the resistance mutation acts in a recessive or semi-dominant manner, mutation of both alleles would be necessary to achieve field resistance. This is of course much less likely than a single mutation. However, other diploid pathogens such as PHYTIN have a history of resistance development and the rusts are notorious for overcoming gene-for-gene based resistance, which again requires two alleles to mutate. There appears to be a discrepancy between prediction and experience for rusts that defies explanation. It would appear prudent to remain vigilant for cases of resistance in rusts.

Fungicide risk factors

History has demonstrated that the risk of resistance differs markedly between fungicide groups. Table 6.1 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. 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.

One approach is experimental. 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 ultraviolet or gamma rays, azide or ethyl methanesulfonate. 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 MOR, should resistant

mutants be detected. Other high-risk fungi such as BOTCIN and PHYTIN are also used. And despite the technical difficulties even powdery mildews have been tested.

Laboratory mutants have been found for a large number of fungicides (see http://www.frac.info/, pathogen risk list). In the majority of cases, field mutants have not so far been found. And when field mutants have been found, the genotype of mutants found in the laboratory differs from that found in the field. 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. Firstly, 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. Secondly, it may be that the resistant mutants carry a sufficient fitness penalty that such strains die out.

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 fungicide batch, adjuvants, 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 HGCA in the UK, carry out these trials (see http://www.hgca.com/cms_publications.output/2/2/Publications/On-farm%20information/Fungicide%20activity%20and%20performance%20in%20wheat.mspx?fn=show&pubcon=9243). 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, a large number of farmers' fields that have been treated with fungicides are inspected each year and unusual cases of disease are noted. In the UK this is called Crop Monitor (http://www.cropmonitor.co.uk/). Suspect isolates from these studies can be collected and tested under controlled conditions.

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 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 of 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 CAA fungicides (Grenville-Briggs *et al.*, 2008).

If the MOR is still unknown after all these analyses have been carried out, the newer genomic methods can be applied (Cools and Hammond-Kosack, 2013). With few exceptions, the genome sequences of all major target pathogens have now been determined (for an updated list, see http://www.genomesonline.org). 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. Similarly, 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. These have largely displaced the chip-based technologies. Genes that are expressed at a higher level in mutant compared with wild type, in the absence or especially the presence of the fungicide, will gives clues both to the MOA and the MOR.

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. The durability of chlorothalonil is of particular value. It is currently used as a mixing partner with high-risk fungicides such as QoI both to extend the spectrum but also to decrease the chance of resistance (Hobbelen *et al.*, 2011). Although multi-site inhibitors are severely restricted in their commercial

applications and value, their non-specific MOA has clear advantages over specific target-site fungicides in terms of resistance development.

Single-site fungicides

Fungicides that target a single vulnerable site are more prone to resistance development than multi-site fungicides. 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:

1. Mutations of the target site gene rendering the gene product more insensitive to the fungicide.
2. Overexpression of the target site gene so that the total capacity of the target pathway is not severely affected.
3. Upregulation of efflux pumps such that the internal concentration of the fungicide is kept below a critical level.
4. Detoxification of the fungicide via glycosylation, or other chemical modification. In contrast to herbicides, this MOR is not important in current fungicides.

These factors are illustrated by discussing the six major fungicide classes that have been most significantly affected by resistance.

Methyl benzimidazole carbamates

The benzimidazoles were among the first systemic fungicides to be marketed. They were hailed as a magic bullet and so when resistance appeared it sent shock waves through the industry. Resistance first appeared just 2 years after their introduction.

C. beticola is a leaf spot pathogen and is prevalent in all areas where sugarbeet is grown, but causes commercially significant levels of disease only in regions with warm summers. The speed of disease establishment increases with increasing daily mean temperature. Additionally, the pathogen requires high humidity for infection and is favoured in crops where overhead irrigation is used.

Ideal conditions for the disease occur in northern Greece, where sugarbeet cannot be grown without the use of fungicides. Traditional methods of control used immobile protectant fungicides, notably fentin acetate, but under high disease pressure such products gave inadequate levels of control, especially in sprinkler-irrigated situations where fungicide wash-off from treated foliage occurred.

The benzimidazoles were among the first systemic fungicides to become available to the grower. In 1967, field testing of benomyl against *C. beticola* showed a twofold superiority in control compared with the organotins. Support grew for the replacement

of protectant fungicides with the new systemics, and by 1972 more than 3000 ha were treated exclusively with benomyl.

Previous seasons, 1970 and 1971, had been encouraging with excellent disease control being maintained by benomyl. By July of 1972, however, a catastrophic decline in control was 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 (Table 6.3; Dovas, 1975).

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.

In 1973, 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 *C. beticola*, increased to over 90% in less than 6 weeks, following only two applications of benomyl. Resistant strains were of equivalent fitness to the sensitive strains, in common with other benzimidazole-resistant fungi.

The genetic basis of the resistance was studied using the model fungus *Neurospora* and shown to be a single gene (Borck and Braymer, 1974). The gene was identified as that encoding β-tubulin in the yeast *S. cerevisiae* (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 changes most commonly seen are E198A,G,K or F200Y (see Box 6.1 for an explanation of nomenclature rules describing sequence variations). Indeed, the mutant versions of these genes were used as selectable markers in fungal transformation experiments. This absolutely 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. Indeed, the resistant mutants are so resistant that it is hard to dissolve an inhibitory concentration of the fungicide. Furthermore there appears to be no fitness penalty. The resistant mutants are 100% of the populations in affected species.

Negative cross-resistance to the *N*-phenycarbamate diethofencarb and the new benzamide class of tubulin inhibitors zoxamide has been reported. In these cases, isolates that are resistant to benomyl are sensitive to diethofencarb and zoxamide and vice versa. It may therefore be possible to use these newer fungicides to control the MBC-resistant pathogens. An alternation strategy would seem to have great potential.

Table 6.3. The performance of benomyl and fentin acetate against *Cercospora beticola* in northern Greece, 1970–1972. (From Dovas, 1975.)

	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	100

Box 6.1. Nomenclature for the description of sequence variations. (From den Dunnen and Antonarakis, 2001.)

A standard nomenclature has been developed that allows researchers to quickly and precisely describe nucleotide and amino acid sequence changes 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 indels in genes. Thus the SEPTRI CYP51 amino acid 524 is the homologue of the ERYGH amino acid 509.

Changes at the DNA level use the > sign. So 12T>A means the thymidine at position 12 is converted to an adenosine.

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 at position 143 is changed to glycine. If the amino acid is changed to several different amino acids, 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 is inserted after a tryptophan 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 position 189 (e.g. G189X) would delete the entire C terminus 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

Quinone outside inhibitors

Resistance to QoI fungicides appeared within 2 years of their introduction around 2000 (Bartlett *et al.*, 2002; Gisi *et al.*, 2002). The resistance was first observed in cereal powdery mildew but has since spread to affect many but not all pathogens. Significant examples are SEPTRI, UNCNEC and other powdery mildews. As before, no rusts have developed resistance. RF values are 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. Instead a very consistent pattern emerged whereby the mutation G143A was found in this gene in essentially all of the affected pathogens. In a few cases, the F129L mutation has been found but this is associated with lower RFs. There was complete cross-resistance with all other QoIs but no other fungicide classes. There 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, *S. tenacellus*, has a CytB with different amino acids in this region.

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 very highly conserved and so degenerate primers should amplify a similar-sized region from different species. Comparison of this region in the wheat tan spot pathogen *Pyrenophora tritici-repentis* and the barley net blotch pathogen *P. teres* 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. However only tan spot had the G143A mutation and these had large RFs and were uncontrolled in the field. It seems that the intron in the 143 codon of *P. teres* prevents the selection of the G143A mutation. The nucleotide change needed to alter the codon from G to A alters the splice site such that the mRNA would never be successfully processed. As *CytB* is an essential gene, such mutations would be lethal. In other words, the mutant strain has zero fitness.

This led researchers to quickly scan other target genomes for the 'blessed' intron. Introns have been found in rust mitochondrial genomes, thereby explaining their failure to develop resistance to QoIs. This was also the case in BOTCIN (Yin *et al.*, 2012). The presence and number of introns in various species vary markedly and does 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. 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 chlorothalonil. Azoxystrobin is sold as a mixture with cyproconazole in the product Amistar Xtra; pyraclostrobin is sold as a mix with epoxiconazole in Opera. This both 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.

Succinate dehydrogenase inhibitors

Succinate dehydrogenase is a complex protein within the mitochondrial membrane comprising four subunits, A–D. All four proteins are encoded by nuclear genes. The original SDHIs were carboxin and oxycarboxin, which had a spectrum limited to basidiomycetes. A resistant strain of *Ustilago maydis* was found to harbour a mutation in subunit B-H272L (Broomfield and Hargreaves, 1992). Although SEPTRI was not commercially controlled by carboxin, resistant mutants could be found in the laboratory with two different mutations B-H272Y,L (Skinner *et al.*, 1998). Transformation of this tractable species with the B-272Y version showed conclusively that this mutation conferred the resistance and identified the target site.

Since 2003 a range of other SDHI fungicides has been released. Resistant mutants in species such as BOTCIN and SEPTRI have been found in the field. A number of sites are affected such as B-P225L,F,T as well as B-H272Y,R,L in BOTCIN (Veloukas *et al.*, 2013). The numbering of orthologous amino acids differs slightly between species. The mutations give general cross-resistance. RFs are moderate and early studies indicate that mutants have a significant fitness penalty (Sierotzki and Scalliet, 2013). Hence SDHI fungicides are regarded as medium to high risk. Most released products contain a second fungicide. After MBCs and QoIs, the industry is taking a cautious approach and monitoring resistance closely (Fraaije *et al.*, 2012).

Demethylation inhibitors

Resistance to G1 DMIs has crept up slowly over the last 20 years and is now a serious issue for this group of fungicides. The target site for DMIs is the *Cyp51/Erg11* gene encoding sterol C14-demethylase. DMIs have been 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. Instead, a gradual decline in the efficacy of certain DMIs was observed and 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 the mid-2000s that resistance was linked to genetic changes in field isolates of pathogens (Cools *et al.*, 2006; Cools and Fraaije, 2013). Since then a plethora of studies have been published which detail the pattern of cross-resistance, RFs and the MORs (Cools and Fraaije, 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 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. A factor here is that some species have two or three *Cyp51* genes. Overexpression of one paralogue appears to confer resistance.
3. Non-target site mutations in efflux pump genes.

Explanations for these findings emerged as genomic technologies were applied to the problem (Cools *et al.*, 2006). Changes in sensitivity were associated with genetic changes in the *Cyp51* gene. A very large number of individual mutations were found. Some were never found singly but only in combination with other mutations.

In order to link phenotype to genotype, a yeast expression assay used previously in medical research was employed (Cools *et al.*, 2010). In this assay, the yeast gene encoding a *Cyp51* orthologue is placed under the control of an inducible promoter. A vector with the pathogen *Cyp51* is inserted into the yeast. Expression of the yeast gene is then switched off. If the pathogen *Cyp51* encodes an active enzyme, the yeast cell can grow. If so, the yeast strain is now dependent on the pathogen's *Cyp51* gene for ergosterol biosynthesis. Hence the EC_{50} values of various DMI fungicides can be tested in an isogenic context. This system can therefore be used to link the various mutations in pathogen *Cyp51* to function. It is a reductionist system that excludes the role of any mutations in other genes in the pathogen.

Using this system several mutations in the SEPTRI *Cyp51* gene have been shown to confer resistance to some of the DMIs. Examples are L50S, Y459D, Y461H, D134G, V136A, Y137F, Y461S and S524T, and the two-amino-acid deletion Δ459/460 (Fraaije *et al.*, 2007; Cools *et al.*, 2010, 2011). This work has been linked to field studies that isolated the pathogen from trial sites treated with different generations of DMI. The frequency of different mutations was compared with the fungicide used. Strains that appear in fields treated with a particular fungicide are deemed to be resistant to and selected by that fungicide. Thus it appears that early DMIs such as tebuconazole selected for the Y137F mutation whereas later DMIs such as epoxiconazole and prothioconazole selected for the S524T mutation. Some mutations appear only in combination with others. The mutation I381V also selects for tebuconazole and difenoconazole but counter selects against prochloraz (Fraaije *et al.*, 2007). RFs vary from 1 (i.e. no effect) to 50 between the different DMIs. These mutations are also found in rusts but did not result in field resistance (Stammler *et al.*, 2009). This proves that rusts are not inherently immune to fungicide resistance.

The yeast studies reveal which mutations are capable of complementing the yeast gene (i.e. they generate an active enzyme) and how well the yeast strain grows. Overall it appears that the Cyp51 enzyme cannot change by single steps into forms that both retain full levels of activity and exhibit high levels of resistance. Combinations of mutations have been selected that represent a compromise between these two parameters. Further combinations of these mutations encode genes with even higher RFs and adequate enzyme activity. These combinations of combinations would be highly unlikely to arise from scratch but can accumulate in a stepwise fashion when DMI use is continued despite a noticeable drop-off in efficacy. The solution to this 'escalator of resistance' is presumably to use other MOAs instead of DMIs. In practice that may mean using mixtures and alternations of fungicide MOAs.

Overexpression of the *Cyp51* gene has also been linked to resistance (Cools *et al.*, 2012). This phenotype is linked to insertions in the promoter of the gene. The RFs are in the range of 7–15 and 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.

The *Cyp51* gene is present in one, two or three copies (paralogues) in different species (Hawkins *et al.*, 2014). All species have at least one *Cyp51* and this appears to be an evolutionarily very old enzyme (Kelly and Kelly, 2013). *Rhynchosporium* has three genes and one, *Cyp51A*, is upregulated in DMI isolates (Hawkins *et al.*, 2014). Similarly there are three genes in *F. graminearum* and this explains why *Fusarium* is not well controlled by DMIs because it is necessary to inhibit all three. Each one has a different profile of sensitivity to DMIs, giving it in-built insensitivity to field rates of these fungicides.

Carboxylic acid amides

It was only when studies of resistance to CAA fungicides were concluded that the MOR and the MOA were identified. CAA fungicides are specific to oomycete pathogens and had been suspected of interfering with cell wall biosynthesis. Resistance was detected in PLASVIT within 2 years of use but had not been detected in PHYTIN even after prolonged use. The resistant PLASVIT mutants were cross-resistant to all CAA fungicides, mandipropimad, dimethomorph and iprovalicarb, indicating a target site mutation. Laboratory PLASVIT resistant mutants were crossed with the wild type (Gisi *et al.*, 2007). Genetic mapping focused attention on the cellulose synthase gene *CesA3*. This identified the MOA. Single nucleotide polymorphisms in the gene segregating with resistance identified the MOR (Blum *et al.*, 2010). The mutation G1105S required two nucleotide substitutions. Resistance to CAA fungicides is regarded as moderate risk mainly because of the features of the target organisms. A resistance management plan is in place.

Acylalanines

The acylalanines are specifically active against oomycete fungi. Acylalanines inhibit RNA biosynthesis through their interference of the activity of a nuclear, α-amanitin-insensitive RNA polymerase–template complex.

The repeated use of (and dependence on) metalaxyl, applied in the field to provide growers with flexible control of downy mildews, established a continuous and high selection pressure that favoured the development of resistance. Resistant strains spread very rapidly. Some cases of resistance in PHYTIN on potatoes, PLASVIT on grapevine, *Pseudoperonospora cubensis* on cucumbers and *Peronospora hyoscyami* f. sp. *tabacina* on tobacco developed within a single season.

In 1984, it was shown that nucleic RNA polymerase isolated from a metalaxyl-sensitive strain of *P. megasperma* f. sp. *medicaginis* could be partially inhibited by metalaxyl, whereas the RNA polymerase from a similar isolation using a metalaxyl-resistant strain was unaffected (Davidse *et al.*, 1984). The mechanism of resistance, therefore, is associated with a mutational change in one of the RNA polymerases. However the mutation responsible for the resistance has not been identified.

The Management of Resistance

Fungicide resistance is now recognized as a fact of life for the fungicide industry. Therefore a 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; see http://www.pesticides.gov.uk/guidance/industries/pesticides/advisory-groups/Resistance-Action-Groups/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 alternative 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, although it is clear that this normally applies to all fungicides with the same MOA. 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 hygiene

Several of the pieces of advice aim to reduce the total amount of the pathogen in the environment of the crop. Thus Advice #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.

Integrated disease management

Advice #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 have to 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 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, 2002, 2003). 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 20 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 disease (or pest) management. IDM (or IPM) embodies the advice that all control methods should be applied simultaneously. 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.

Dose rate

Advice #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 #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 (and hence nuclei) 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 has been the subject of intense debate (Shaw and Pijls, 1994; Zziwa and Burnett, 1994). It is now established for the great majority of cases that the lower the dose the lower the risk of resistance. This result is supported by both modelling and experience (Van den Bosch *et al.*, 2011). Rationalization of this finding stems from the simple idea that the resistant isolates of the pathogen survive with higher frequency at all doses of the fungicide (Fig. 6.5). In Fig. 6.5 the selection pressure is represented by the vertical arrows and is higher at higher doses.

Figure 6.5a models a fungicide resistance with a moderate RF. Figure 6.5b represents a high RF; the selection pressure still increases with increasing dose. Figure 6.5c represents a fungicide resistance with a significant fitness penalty. Here the selection pressure is negative at low doses and increases with dose. Figure 6.5d represents a situation not yet seen in fungi but seen in weeds where the survival frequency converges at very high doses. In this case the selection pressure varies both up and down with dose.

The concept that low dose equates to low risk was counterintuitive and contrary to the advice for herbicide resistance. 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 prior warning of resistance occurs.

More important, however, is the effect of ploidy. Weeds are normally diploid and most herbicide resistance traits are semi-dominant. So if one allele of a herbicide tolerance gene

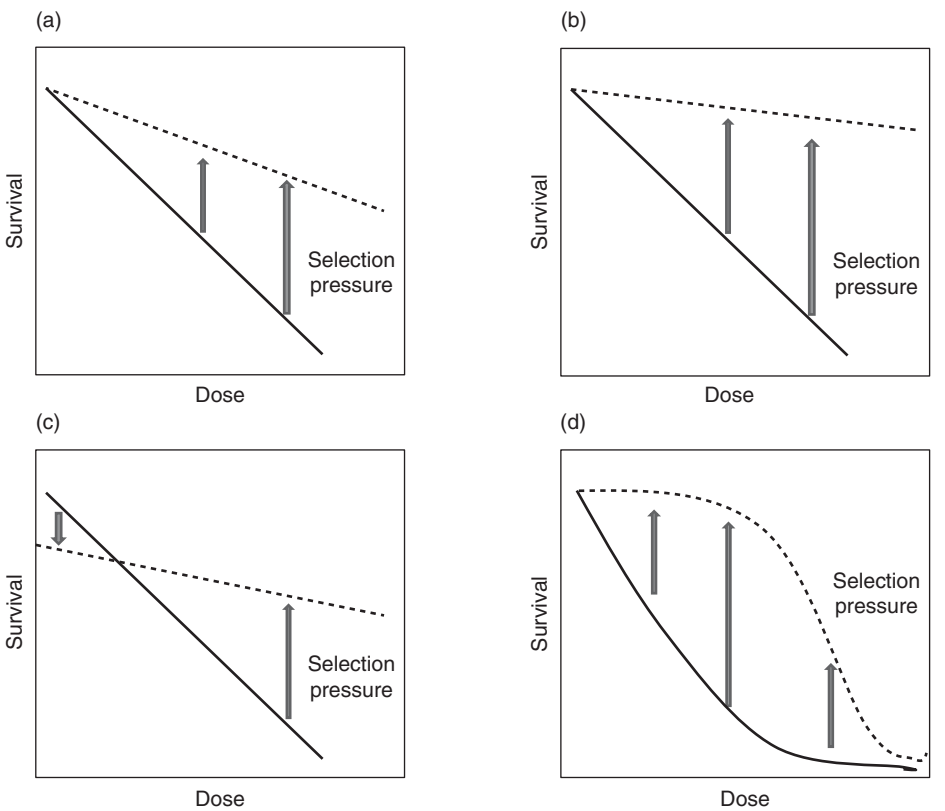


Fig. 6.5. Schematic dose response curves for wild type (——) and resistant mutant (– – –). Panel (a) represents a mutant with a moderate resistance factor (RF) and shows that the selection pressure (vertical arrow) is higher at higher doses. Panel (b) shows a mutant with a high RF; the selection pressure 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.

mutates, this heterozygous plant would survive a moderate dose, higher than the homozygote sensitive but lower than homozygote resistant. The chances of both alleles mutating 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 (but not all) pathogens are haploid and so the concept of heterozygous resistance does not apply.

Mixtures and alternation

Advice #9 argues against the repeated use of the same MOA. Accordingly Advice #6 advises using either mixtures or alternation with different MOAs. At a simplistic level, it is easy to rationalize that using different fungicides in either alternations or mixtures would delay the emergence of resistance. Repeated use of the same fungicide MOA applies the selection pressure repeatedly to the already selected population. Regulatory authorities therefore legislate for the maximum number of times an MOA can be used in a given period.

Mixtures or alternations should be a good way to prevent resistance (Hollomon and Kendall, 1997). 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; for example of QoI and DMIs, or QoIs and chlorothalonil. On the other hand, use of a mixture might be thought to promote the selection of mutants resistant to both the fungicides. This has so far not been observed (Hobbelen *et al.*, 2013; Spolti *et al.*, 2013). Mixtures of DMIs may provide protection as different DMIs seem to select different mutations (Fraaije *et al.*, 2007).

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 (e.g. QoI) and low risk (e.g. 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 #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.

It might be argued that there is a higher risk of developing resistance to both fungicides, either by selection of pump-based resistance or of both single-site mutations. However no cases of this scenario have so far been detected.

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. 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.

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