Hideo Ishii · Derek William Hollomon
Editors

Fungicide Resistance in Plant Pathogens

Principles and a Guide to Practical Management

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Preface

Modern fungicides have greatly contributed to the control of crop diseases. However, the development of resistant fungal strains has caused a failure of disease control by many fungicides. By the early 1970s, resistance became a practical problem in many countries, and extensive research has been done since then. Dekker and Georgopoulos (1982), the two pioneer scientists in fungicide resistance, published the first textbook on this subject, but it is now timely to update our understanding of the principles underpinning sound resistance management.

Nearly 50 years have passed since we first recognized the importance of fungicide resistance. Since then, the Fungicide Resistance Action Committee (FRAC) established by manufacturers, public organizations including the Fungicide Resistance Action Group in the UK (FRAG-UK), and similar groups in many other countries and learned societies such as the Phytopathological Society of Japan all exist to alert users to resistance problems and advise on anti-resistance management strategies. Nevertheless, resistance remains a serious problem and can emerge rapidly.

In recent years, the development of new fungicides has become more difficult as increasing amounts of environmental and toxicological data are needed to satisfy regulatory authorities. In addition, many existing fungicides may be banned in the near future due to suspected toxicological reasons. Concern over the loss of key modes of action was expressed in the Declaration of Ljubljana, which states that "In order to safeguard the production of food at affordable prices, it is essential to provide farmers with access to sufficient diversity of crop protection solutions. This is essential to prevent or delay the development of resistant pests, and to maintain the efficacy of remaining crop protection products" (Bielza et al. 2008).

Against this background, the editors proposed publishing an updated text on resistance for students and researchers because we believe that regulation based on the precautionary principle involving hazard rather than scientific-based risk assessment of fungicide use will reduce the diversity of modes of action and increase resistance in the near future. To manage fungicide resistance successfully will require the promotion of integrated disease management, involving not just chemical

fungicides but also host plant resistance, agronomic factors, and reliable biological control agents where these are available.

This book comprises four parts: Development of Fungicide Resistance, Mechanisms of Resistance, Monitoring Resistance, and Resistance Management in Major Crops. In total, 29 chapters have been written by representative scientists in this field worldwide. The chapters cover the most important fungicide groups that have caused resistance on various crops. This book includes descriptions of the basics of fungicide resistance including the history, genetics, evolution, and also up-to-date information on mechanisms and management of resistance.

It is a great pleasure for the editors to draw on their experience to create a book that we believe will help readers understand more about fungicide resistance and its management. We must also take this opportunity to thank Ms. Fumiko Yamaguchi and Dr. Mei Hann Lee, from Springer Japan, both for their assistance in editorial matters and for overseeing the production of the book.

Minami-Awaji, Japan — Hideo Ishii
Bristol, UK — Derek William Hollomon

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Contents

Part I
Development of Fungicide Resistance

Chapter 1
Fungicide Resistance: 40 Years on and Still a Major Problem

Derek William Hollomon

Abstract Fungicide resistance emerged as a practical disease control problem in the 1970s, but it was the outcome of two workshops in Wageningen in 1980 and 1981 that set the framework for research to tackle the problem. Some but not all fungicides quickly select already-existing resistant mutants from within target pathogen populations. Several mechanisms contribute to resistance, but where target-site changes predominate, cross resistance does not extend to other modes of action. Field efficacy and bioassay are key to confirming resistance, but molecular techniques are increasingly used to detect resistance and to augment biochemistry to determine mechanisms. Resistance is not inevitable but depends on the impact of both pathogen and fungicide properties on pathogen populations. Some factors can be manipulated to minimise resistance risk, and a cornerstone of anti-resistance strategies combines treatments with more than one mode of action, either in mixtures or in alternation. Controlled release formulations may also help reduce selection. Resistance has a financial cost to users and manufacturers and seriously reduces available modes of action. Consequently to combat resistance, fungicides should be embedded in integrated disease management systems.

Keywords Cereal eyespot • Controlled release formulations • Evolution of resistance • Fungicides • Griseofulvin • Monitoring

1.1 Introduction

My interest in fungicide resistance began whilst at Rothamsted in the 1970s, when resistance was emerging as a serious disease control problem. Workshops at Wageningen in 1980 and 1981 strengthened the resistance agenda, resulting in the establishment of the industry-based Fungicide Resistance Action Committee (FRAC) and in the publication of a book based on the workshop's proceedings, edited by Johan Dekker and Spiros Georgopoulos (1982). This book quickly became the standard text for those involved in fungicide resistance. Inevitably things have

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moved on since the early 1980s. A glance through the book's pages finds no mention of molecular techniques which now impact greatly on resistance research, especially in the areas of mode of action, resistance mechanisms and monitoring. Epidemiologically based modelling of the evolution of resistance and anti-resistance management strategies have also expanded greatly since the 1980s. To illustrate these changes I have included some key references in this chapter that have shaped the direction of resistance research over the past 40 years. This helps set the scene for the chapters that follow and which provide an up-to-date account of where we are today in dealing with the resistance problem.

1.2 History

Chemistry has a long history of involvement in disease control. The use of lime sulphur sprays for powdery mildew control became popular from 1800 onwards (Forsyth 1802), but the discovery of Bordeaux mixture by Millardet (1885) and use of copper compounds for control of grape downy mildew (*Plasmopara viticola*) and potato late blight (*Phytophthora infestans*) marked the beginning of fungicide research within the agrochemical industry. It prompted research that eventually led to the introduction of organic compounds and especially dithiocarbamates (thiram, mancozeb), phthalimides (captafol) and chlorothalonil, which are now very widely used as sprays or seed treatments throughout the world. Despite extensive use over many years, resistance has not been a problem with these largely nonsystemic protectant fungicides because of their multisite modes of action.

A serious limitation of protectant fungicides is that they hardly penetrate into plants, and so established infections are not controlled, and losses through weathering coupled with the need to protect new foliage require frequent applications, often at weekly intervals. A landmark step in overcoming this problem was the demonstration that the antifungal antibiotic (griseofulvin) isolated from *Penicillium griseofulvum* was translocated within plants and not only controlled established infections but protected new growth (Brian et al. 1951). Unfortunately its high cost prevented use in crop production, although it found use as a medical fungicide. Nevertheless, it illustrated that systemic protection was possible and stimulated the search for novel systemic compounds, many of which are now key components in the successful management of otherwise damaging crop diseases. Because systemic fungicides necessarily have a close association with the biochemistry and physiology of plants, their modes of action are specific and usually involve just one biochemical target site.

To date 42 different modes of action (FRAC 2014) are identified for fungicides.

1.3 Evolution of Resistance

Darwin viewed all organisms as survivors which, through natural selection, competed with others in their particular environment (Darwin 1859). Fungicides disrupt metabolism and threaten survival, so it is no surprise that pathogens can

initiate mechanisms to resist lethal effects. Fungal genomes are very plastic and may contain many thousands of polymorphisms (Cuomo et al. 2007). Large pathogen populations will inevitably contain rare distinct genetic, and stable, individuals able to counter to varying degrees any unfavourable metabolic impact and increase in response to selection at the expense of sensitive components of the population. What form resistance will take depends on the resistance mechanism selected. Where the fungicide target is a specific biochemical step, a single point mutation causing one amino acid change can rapidly and effectively block fungicide binding within the target site (single-site inhibitors) and generally causes high levels of resistance. Fungicides that target many biochemical steps (multisite inhibitors) require a combination of many mutations and so resistance evolves slowly, if at all.

An effective resistance mechanism does not ensure that a practical disease control problem will evolve. In treated crops resistant individuals will be more fit than the wild-type sensitive population. But initially, in the absence of the fungicide, resistant individuals must have a lower relative fitness; otherwise, any new fungicide would not offer control benefits. Although any fitness penalty may be linked to decreased enzyme efficiency inherent in the target-site change (Nicholas et al. 2004), many environmental factors also influence fitness, and mutations can alter the general genetic background so that resistant individuals are no longer at a fitness disadvantage.

A core feature of fungicide resistance is that products with the same mode of action, and hence the same specific resistance mechanism, show cross resistance, but not resistance to other modes of action. Active efflux mechanisms may also contribute to resistance. This phenomenon known as multidrug resistance (MDR) may generate resistance between products with different modes of action, especially in laboratory assays. However, resistance levels are low, perhaps up to 20-fold in some pathogens (Kretschmer 2012), so in practice MDR only augments target-site resistance.

But even products in the same mode of action group (FRAC 2014) may interact somewhat differently to a particular change in the target site, resulting in differences in resistance levels which in turn impact on the evolution of resistance within pathogen populations. Prothioconazole, for instance, interacts with the haem component of the target-site sterol 14α-demethylase (CYP51) differently from other azoles (Parker et al. 2011), showing lower resistance and still effective control of some cereal diseases. Extensive analysis of azole resistance in *Mycosphaerella graminicola*, the cause of wheat leaf blotch, has shown that different target-site mutations alone, or in combination, generate different cross resistance patterns (Cools et al. 2011) and indeed improved the performance of prochloraz (Leroux and Walker 2011). A structural analysis of the impact of these CYP51 changes on azole sensitivity provides a potential insight to manage resistance through new chemistries (Kelly and Kelly 2013).

1.4 Detecting Resistance

Resistance above all is a field-based problem recognised by a decline in fungicide performance, to which growers may often respond by increasing dose rate and/or treatment frequency. Poor performance can be caused by a host of factors, including poor application and timing, wrong dose rate or very exceptional disease pressure. So anecdotal evidence from growers must be backed up by a programme of field work supported by glasshouse and laboratory assays.

Development of a new fungicide involves many efficacy trials involving different dose rates and carried out under a range of environmental conditions. If resistance is a problem, repeating these field trials should show a decline in performance. But this approach to confirming resistance requires more than just a single season's work.

A more common approach to confirming resistance involves comparing the sensitivity of isolates obtained from sites where performance has eroded with the sensitivity of isolates never exposed to the at-risk fungicide. Underpinning this approach is the need to have developed suitable bioassays in which there is a clear relationship between dose rate and response which, depending on the pathogen and the fungicide mode of action, may involve measuring germination, germ-tube or mycelial growth rate or, especially for obligate pathogens, infection levels. Ideally the existence of a sensitivity distribution of the target fungal population established prior to widespread use of a new fungicide will allow a meaningful confirmation of resistance. The key role of a "baseline" sensitivity distribution in various aspects of resistance management was discussed in detail by Russell (2003), and its importance is recognised in many countries where a baseline sensitivity distribution is a requirement for registration of a new fungicide. The ability to confirm resistance through comparison with a baseline sensitivity will depend on the sample size from the suspected resistant population and inclusion of at least one reference isolate to check for variation between assay tests. In practice where baseline sensitivity data do not exist, comparisons can be made between isolates obtained from at-risk sites with those collected from untreated areas. Often researchers obtain baseline data using "historic" isolates which have been maintained in culture collections, sometimes for many years, and which were isolated before the at-risk fungicide was used.

The first attempts to diagnose fungicide resistance using molecular techniques were reported in the early 1990s and involved monitoring benomyl resistance (Koenraadt and Jones 1992; Koenraadt et al. 1992). Since then tremendous advances have been made in polymerase chain reaction (PCR) and sequencing technologies, and which now allow rapid detection of single-nucleotide polymorphism (SNP) mutations causing resistance. Indeed the literature is full of different molecular techniques used to monitor resistance, and certainly the most well documented is perhaps detection of the mutation generating the G143A amino acid change in the target b-type cytochrome of complex III of respiration, causing resistance to QoI fungicides (Di Rago et al. 1989).

However, molecular techniques are only useful after resistance has been confirmed using bioassays, the resistance mechanism determined and the DNA change causing resistance identified. But ample evidence suggests that a target-site change causing resistance in one pathogen will occur in other pathogens, so molecular techniques are being used to monitor for resistance in pathogen populations that have not yet evolved resistance in the field to a particular mode of action. Molecular technologies present a different concept of "baseline" from that understood from bioassay data. Resistance can be defined in terms of the frequency of the resistance-causing mutation compared with wild-type frequency, but it is not necessarily clear what frequency of the resistance mutation will cause disease control problems in the field. Indeed, molecular techniques can be extremely sensitive, detecting perhaps 1 in 10,000 mutations in target populations that are clearly sensitive (Windass et al. 2000). Equally relevant is that other point mutations may cause resistance (eg. F129L and G137R Leadbeater 2012) in the case of QoI resistance, and which requires a battery of molecular assays where one bioassay would suffice. Furthermore, in diploid or polyploid *Oomycetes* "fungi", mutations may be recessive (Gisi et al. 2007) and, therefore, simply detecting a mutation may not be sufficient to confirm resistance.

A final step towards confirming resistance requires rigorously conducted *in planta* assays involving a range of dose rates, preferably using single spore isolates, and comparing a wild-type isolate with at least one suspected of being resistant. Generating a dose–response relationship will provide not only measure of sensitivity of each isolate (commonly the effective dose needed to reduce infection 50 %, i.e. EC_{50}) but also a resistance factor (RF).

A detailed characterisation in this way of one or more resistant isolates obtained from the field provides a platform to determine the biochemical and molecular mechanism of resistance, which may not be the same as mechanisms identified in resistant mutants generated in the laboratory during the development programme. It also provides standard resistant isolates available for use in monitoring surveys and for other research programmes.

1.5 Likelihood of Resistance

The likelihood of resistance is the outcome of the impact of fungicide treatment on the target population and depends on both biological and chemical factors. Many of the pathogen, or intrinsic, properties (Table 1.1) contribute to the "pathogen risk" and are mostly outside the control of the grower. But many of the treatment measures (Table 1.2) provide opportunities for growers to adjust the risk of resistance for a particular pathogen/fungicide combination.

Historically, resistance emerged quickly where growers were cultivating disease-susceptible crops (especially cereals, cucurbits and vines) and relying extensively and repeatedly on fungicides with a single mode of action to control disease. Choosing less-susceptible cultivars where possible and operating an integrated

Table 1.1 Pathogen properties influencing evolution and spread of resistance

Biochemical
Dependence on disruptible biochemical steps
Availability of resistance mechanisms
Epidemiological
Dispersal method, e.g. wind, rain splash, soilborne
Abundance of sporulation
Pathogen life cycle: short or long generation time
Ability to infect all crop stages, requiring repeated treatment
Isolation of pathogen populations preventing re-entry of more competitive sensitive genotypes
Genetic
Relative abundance of genotypes with different sensitivities
Fitness properties of different genotypes
Sexual or asexual reproduction: influence on inheritance of resistance
Mutation rate
If relevant dominance of resistance alleles

Table 1.2 Fungicide properties influencing evolution and spread of resistance

Biochemical
Interaction with target metabolism and its susceptibility
Physicochemical/toxicological
Stability, solubility, volatility, polarity
Partition and transport properties
Application
Initial dose and distribution
Formulation
Exclusive and repeated use of at-risk mode of action
Extent of area treated
Integration with other disease management tools, including biofungicides, resistant varieties, crop rotation and crop hygiene

disease management (IDM) programme employing different modes of action, either in mixtures or in alternation, has become the cornerstone of anti-resistance management systems. Kable and Jeffrey (1980) were perhaps the first to employ a modelling approach to management of fungicide resistance. More recent publications discuss in detail the assessment and management of resistance risk (Kuck 2005; Brent and Hollomon 2007a, b).

One question that occurs repeatedly in discussions with growers about resistance risk relates to the impact of the dose rate (van den Bosch et al. 2011). A factor which receives little attention in this debate, but which could impact significantly on the likelihood of resistance, involves how pathogens are actually exposed to fungicides. Ideally, fungicide doses should be just sufficient to kill enough of the wild-type population to provide acceptable control levels. In practice, because of the exponen-

tial kinetics of decay due to evaporation, metabolic degradation in both the host plant and the pathogen and poor rain fastness, initial dose rates are much higher than needed to inhibit pathogen growth. High initial doses, especially in an eradicant mode, certainly imply a high selection pressure and, depending on the range of rare resistant individuals in the population, could increase the likelihood of resistance. One way to reduce initial dose rates would be to use controlled release formulations which maintained a lower but steady dose rate, over a longer period of time. It suggests that more attention needs to be given to formulation in the management of resistance and how it could be used to achieve a gradual release of an active ingredient held in the leaf surface. A somewhat old (but very relevant) discussion of formulation in relation to efficacy was given by Graham-Bryce (1987), and Shephard (1985) presented evidence that release of different azoles from reservoirs in the cuticular leaf layers depends on formulation,

1.6 What Does Resistance Cost?

Resistance does not come without a cost to both growers and manufacturers. However, useful economic data are seldom available, especially from naturally infected field or glasshouse trials where losses can be accurately quantified. Although costs will probably be similar for other cereal diseases, my own experience is from a series of trials in the 1980s, following failure of MBC (carbendazim, benomyl) fungicides to control cereal eyespot (*Oculimacula yallundae*) because of resistance. Without treatment eyespot causes wheat losses of 10 %, whilst MBCs reduced losses to 3 % (Pavely et al. 2011). At current wheat prices (£120/ton), this failure to control eyespot equates to a loss of £170 m per annum for UK growers, compared with a £49 m loss before carbendazim resistance. Fortunately an alternative, but more expensive, eyespot fungicide (prochloraz) was available in the 1980s; otherwise, without effective eyespot control, wheat would have been an unprofitable crop for some growers. Similar economic losses no doubt occurred as a result of phenylamide resistance in *Phytophthora infestans*, the cause of late blight of potato.

Although manufacturers' losses are equally serious, no detailed costs are available. Registration authorities have responded to resistance by requiring additional data before giving approval for new products (Kuck 2005). Not only are baseline sensitivity distributions needed for target pathogens but also information on mode of action, cross resistance, assessment of resistance risk and proposed anti-resistance management strategies. To generate all this information requires a substantial commitment of resources, not only within the development programme for a new product but also in support of an existing product when its use increases as a replacement for fungicides no longer effective because of resistance. Add to this loss of sales revenue, coupled with redirecting resources to monitoring resistance, stewarding product use, and adapting chemical plant for other uses, leaves less available for research and development of products with new modes of action. This last point emphasises a major cost of resistance, not just for growers and manufacturers but

also for consumers, in so far as resistance reduces the modes of action available to combat the problem. To respond to these many challenges, resistance management teams have been expanded in all the major manufacturing companies.

1.7 Future Directions

The following chapters amply illustrate that, whilst significant progress has been made in understanding and managing fungicide resistance, the problem remains. Despite the fact that effective anti-resistance management requires access to different modes of action, governments, and especially the European Union, continue to enact legislation, without much scientific evidence, that reduces the number of modes of action, including possibly azoles! Against this background future anti-resistance strategies will be embedded in integrated disease management (IDM) systems, which combine conventional chemical fungicides with biofungicides and host plant resistance, generated either by conventional plant breeding or by GM technologies. To what extent biofungicides (usually bacteria and fungi) offer new modes of action is unclear, although the ease with which it is now possible to follow changes in expression levels of genes involved in the activation of host resistance suggests that the mode of action of some may involve systemic acquired resistance (SAR). Evidence from the long-term and effective use of the rice blast fungicide probenazole which activates SAR suggests this mode of action is not easily overcome by the development of resistance. But whatever the modes of action of biofungicides, it is quite possible that pathogens will eventually evolve resistance to them. Finally, complex IDM strategies will challenge pathologists to define treatment thresholds and monitor changes in pathogen populations and will challenge growers to maintain production and profitability.

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Chapter 2
Genetics of Fungicide Resistance

Rita Milvia De Miccolis Angelini, Stefania Pollastro, and Franco Faretra

Abstract Acquired resistance to fungicides in fungal plant pathogens is a challenge in modern crop protection. Fungi are indeed very able to adapt to changing environmental conditions, such as the introduction of a new fungicide in the agricultural practice. Several genetic mechanisms may underlay fungicide resistance and influence the chance and time of its appearance and spreading in fungal populations. Resistance may be caused by mutations in major genes (monogenic or oligogenic resistance) or in minor genes (polygenic resistance) which may occur in nuclear genes as well as in cytoplasmic genes. They are immediately expressed in haploid fungi, while they may be dominant or recessive in diploid fungi. Allelic variants may cause different levels of resistance and/or different negative pleiotropic effects on the fitness of resistant mutants. The sexual process, where occurring, plays an important role in releasing new recombinant genotypes in fungal populations. Heterokaryosis provides multinucleate fungi with a further mechanism of adaptation. Resistant mutants can be obtained from samples representative of field population of a pathogen or under laboratory conditions through selection of spontaneous mutations or following chemical or physical mutagenesis. Nowadays, molecular tools, such as gene cloning, sequencing, site-directed mutagenesis and gene replacement, make genetic studies on fungicide resistance amenable even in asexual fungi for which classical genetic analysis of meiotic progeny is not feasible.

Keywords Mutations • Major genes • Minor genes • Cytoplasmic genes • Ploidy • Heterokaryosis • Population genetics

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2.1 Introduction

Resistance to chemicals in microorganisms is a very common phenomenon occurring whenever antimicrobial compounds are used against pathogens of plants, animals or humans. Natural or innate resistance refers to intrinsic features (e.g. the lack of a specific molecular target and/or a metabolic pathway) protecting the organism from the effects of antimicrobials. For example, strobilurin-producing organisms, including wood-degrading *Basidiomycetes*, such as *Strobilurus tenacellus*, have innate resistance to their own strobilurins that show, instead, activity against a very broad spectrum of fungi and *Oomycetes*. Acquired resistance refers to organisms that in their wild-type form are sensitive and may develop resistance after their exposure to an antimicrobial compound. Acquired resistance is due to genetic modifications transmissible to the progeny so that a chemical that was once effective against the organism is no longer effective.

Resistance to fungicides used in agriculture as well as in animal or human health care is a more recent phenomenon than resistance to antibiotics (Coplin 1989; Cookson 2005) and insecticides (Brown 1977). Until the late 1960s, fungicides used in crop protection (e.g. sulphur, copper derivatives, dithiocarbamates) were indeed essentially multisite inhibitors, affecting multiple target sites and hence interfering with many metabolic processes of the pathogen. Despite their protracted and widespread use, acquired resistance to multisite fungicides is still a rare event. This is because there is a low probability that a number of mutations at different loci, needed for the onset of the resistance, simultaneously occur in fungal cells and, if this happens, the mutated isolates remain viable. Afterwards, with the introduction of single-site fungicides and as a consequence of their frequent and repeated use, fungicide resistance has become a major concern in modern crop protection seriously threatening effectiveness of several fungicides (Brent and Hollomon 2007a, b).

Fungicide resistance is hence a result of adaptation of a fungus to a fungicide due to a stable and inheritable genetic change, leading to the appearance and spread of mutants with reduced fungicide sensitivity (Delp and Dekker 1985).

2.2 Genetic Bases of Fungicide Resistance

Genetics of fungicide resistance have been previously reviewed by Grindle (1987), Grindle and Faretra (1993), Steffens et al. (1996) and Ma and Michailides (2005), and deeper information is available on the website of the Fungicide Resistance Action Committee (www.frac.info).

Fungal genetic backgrounds and genetic bases of resistance are key factors in the intrinsic risk of resistance and influence its evolution in the pathogen populations. For example, the occurrence of genetic recombination through the sexual process, where it regularly occurs in nature, or parasexuality, in essentially asexual fungi, may greatly influence the dynamics of resistant subpopulations

producing new combinations of resistance and fitness traits originally occurring in separate individuals.

Most genetic studies on fungicide resistance have been carried out on 'model' saprophytic *Ascomycetes*, such as *Aspergillus nidulans*, *Neurospora crassa* and *Saccharomyces cerevisiae*. Nevertheless, the genetics of fungicide resistance has been investigated in several pathogenic fungi (Table 2.1).

Key factors in the genetic bases of fungicide resistance are (1) the number of loci involved, (2) the number of allelic variants at each locus, (3) the existence and relevance of dominant or recessive relationship between resistant and wild-type alleles (Borck and Braymer 1974) and (4) the additive or synergistic interactions between resistance genes.

Genes responsible for fungicide resistance may be located on chromosomes inside the nucleus or on extrachromosomal genetic determinants. Nuclear and cytoplasmic genes can be distinguished by their inheritance patterns. Nuclear genes typically show classical biparental (disomic) inheritance in sexual crosses, i.e. the zygote receives one allele of each gene from each of its parents. In contrast, genetic material in the cytoplasm has a non-Mendelian inheritance and is characterized by uniparental (usually maternal) transmission (Griffiths 1996). In addition, cytoplasmic genes differ from nuclear genes in showing vegetative segregation and intracellular selection potentially affecting resistance stability (Birky 2001; Ziogas et al. 2002).

Most fungicide-resistance genes are located on nuclear chromosomes. In most cases, there is only one copy of resistance gene in the genome and mutations are usually located in gene sequences encoding enzymatic or structural proteins. However, multidrug resistance (MDR) in *B. cinerea* and other fungi is caused by overexpression of membrane efflux transporter genes resulting in an increased efflux of toxicants that reduces fungal sensitivity to several unrelated fungicides as well as plant defence chemicals (reviewed by Kretschmer 2012). In MDR1 strains of *B. cinerea*, resistance is conferred by mutations in the regulator *mrr1* gene encoding a transcription factor controlling the ABC transporter *AtrB* gene, whereas in MDR2 strains resistance is caused by an insertion of a retrotransposon-derived sequence in the promoter region of the facilitator superfamily (MFS) transporter gene *mfsM2* (Kretschmer et al. 2009).

Fungicide resistance may result from mutations in single major genes (Georgopoulos 1988) or from additive (Kalamarakis et al. 1991; Lasseron-de Farandre et al. 1991) or synergistic interactions (Molnar et al. 1985) between several mutant genes.

Monogenic and oligogenic resistance are caused, respectively, by one or few major genes. Major genes have an appreciable influence on the phenotype, and resistance mutations cause a qualitative change in the response to a fungicide with the appearance in the field of new fungicide-resistant subpopulation(s) well distinguishable from the wild-type sensitive one (Fig. 2.1). Most cases of fungicide resistance are due to mutations in major genes (Table 2.1). Mutations in major genes conferring resistance to fungicides having different modes of action may also occur in a same isolate, causing multiple resistance. In oligogenic resistance, several different major genes are involved, any one of which can mutate to cause an increase

Table 2.1 Most relevant examples of the genetic bases of resistance to fungicides in fungal plant pathogens

Fungicides (chemical groups)	Mode of action and target site	Genetic bases of the resistance	Target site mutations	References
Benzimidazoles and thiophanates [MBC (methyl benzimidazole carbamates)]	Mitosis and cell division: ß-tubulin assembly in mitosis	Monogenic resistance due to mutations in single major genes. Multiallelic resistance. Negative cross resistance between benzimidazoles and *N*-phenylcarbamates observed in *Botrytis cinerea* or between MBCs and the fungicides diethofencarb and zoxamide in *Pyrenopeziza brassicae*	Several SNPs, mostly E198A/G/K, F200Y in β-tubulin gene (e.g. *Mbc1* gene in *Botrytis cinerea*)	Faretra and Pollastro (1991), Yarden and Katan (1993), Nakazawa and Yamada (1997), and Ishii (2012b)
N-Phenylcarbamates				
Dicarboximides	Signal transduction: MAP/osmosensing class III histidine kinase	Monogenic resistance due to mutations in single major genes. Multiallelic resistance	Several mutations in *os-1* (*Daf1*) gene, mostly I365S or different substitutions in conserved amino acid domains	Faretra and Pollastro (1991), Cui et al. (2002), Oshima et al. (2002), Yoshimi et al. (2003), Dry et al. (2004), and Fillinger et al. (2012)
		Hypersensitivity to high osmolarity frequently associated to the resistance		
PP fungicides (phenylpyrroles)	Signal transduction (mechanism speculative): MAP/osmosensing class III histidine kinase	Monogenic resistance due to mutations in single major genes also conferring resistance to dicarboximides and increased osmotic sensitivity. Additional mechanisms (i.e. overexpression of efflux transporters in MDR mutants of *B. cinerea*)	Mutations in osmosensing class III histidine kinase genes (*os-1*; *os-2*, *HOG1*)	Faretra and Pollastro (1993b), Ochiai et al. (2001), Zhang et al. (2002), Avenot et al. (2005), and Fillinger et al. (2012)

PA fungicides (phenylamides)	Nucleic acid synthesis: RNA polymerase I	Monogenic resistance based on a single incompletely dominant gene or both a major and several minor genes	Unknown	Shattock (1988), Crute and Harrison (1988), and Hermann and Gisi (2012)
SDHI (succinate dehydrogenase inhibitors)	Respiration: complex II (succinate dehydrogenase)	Monogenic resistance due to mutations in single major genes. Multiallelic resistance. Partial cross resistance between SDHIs	SNPs in SDH genes (*SdhB*, *SdhC* and *SdhD*), e.g. H/Y (or H/L) at 257, 267, 272 or P225L/T/F in *SdhB* gene, dependent on fungal species	Skinner et al. (1998), De Miccolis Angelini et al. (2010a), and Sierotzki and Scalliet (2013)
QoI fungicides (quinone outside inhibitors)	Respiration: complex III: [cytochrome bc1 (ubiquinol oxidase) at Qo site (*cyt*b gene)]	Point mutations in a mitochondrial gene or additional mechanisms (i.e. alternative respiration and efflux transporters)	SNPs in *cyt*b gene, mostly G143A (in several fungal species), F129L or G137R	Gisi et al. (2002), Kim et al. (2003), and Sierotzki et al. (2007)
AP fungicides (anilinopyrimidines)	Amino acids and protein synthesis: methionine biosynthesis (proposed) (*cgs* gene)	Monogenic resistance due to mutations in single major genes. Alternative mechanisms (i.e. overexpression of efflux transporters in MDR mutants of *B. cinerea*)	No mutations associated with AP resistance in *cbl*, *cgs* or other key genes involved in the biosynthesis and metabolism of methionine or sulphate assimilation	Hilber and Hilber-Bodmer (1998), Chapeland et al. (1999), De Miccolis Angelini et al. (2010b), and Liu et al. (2014)

(continued)

Table 2.1 (continued)

Fungicides (chemical groups)	Mode of action and target site	Genetic bases of the resistance	Target site mutations	References
DMI fungicides (demethylation inhibitors) SBI: class I	Sterol biosynthesis in membranes: C14-demethylase in sterol biosynthesis (erg11/cyp51)	Mostly polygenic resistance (mutations in unlinked genes which show an additive effect). Resistance also conferred by single major genes (monogenic, e.g. in *Erysiphe necator*) or by few resistance genes (oligogenic, e.g. in *Blumeria graminis* f.sp. *hordei*). Additional mechanisms (e.g. ABC transporters)	Mutations in *cyp51* (*erg11*) gene, e.g. V136A, Y137F, A379G, I381V, or in *cyp51* promotor	Kalamatrakis et al. (1991), Peever and Milgroom (1992), Délye et al. (1997), Brown et al. (1992), Blatter et al. (1998), and Cools and Fraaije (2013)
Amines ('morpholines') SBI: class II	Sterol biosynthesis in membranes: Δ14-reductase and Δ8 → Δ7-isomerase in sterol biosynthesis (erg24, erg2)	Polygenic resistance due to mutations in unlinked genes which show an additive effect. No cross resistance to other SBI classes	Unknown	Lasseron-de Falandre et al. (1991) and Markoglou and Ziogas (1999, 2000, 2001)
Hydroxyanilides SBI: class III	Sterol biosynthesis in membranes: 3-keto reductase, C4-demethylation (erg27)	Monogenic resistance due to mutations in single major genes. Additional mechanisms (e.g. P450-mediated detoxification of fenhexamid) have been proposed	Several mutations in *Erg27* gene, mostly F412S/I/V in *B. cinerea* HydR3^{+} strains	Leroux et al. (2002), De Guido et al. (2007), and Fillinger et al. (2008)
CAA fungicides (carboxylic acid amides)	Cell wall biosynthesis: cellulose synthase	Monogenic resistance due to point mutations in a recessive nuclear gene	SNPs in *CesA3* gene leading to amino acid changes in cellulose synthase 3, i.e. G1105A/S/V/W, V1109L/M or Q1077K	Gisi et al. (2007), Blum et al. (2010, 2012), and Pang et al. (2013)

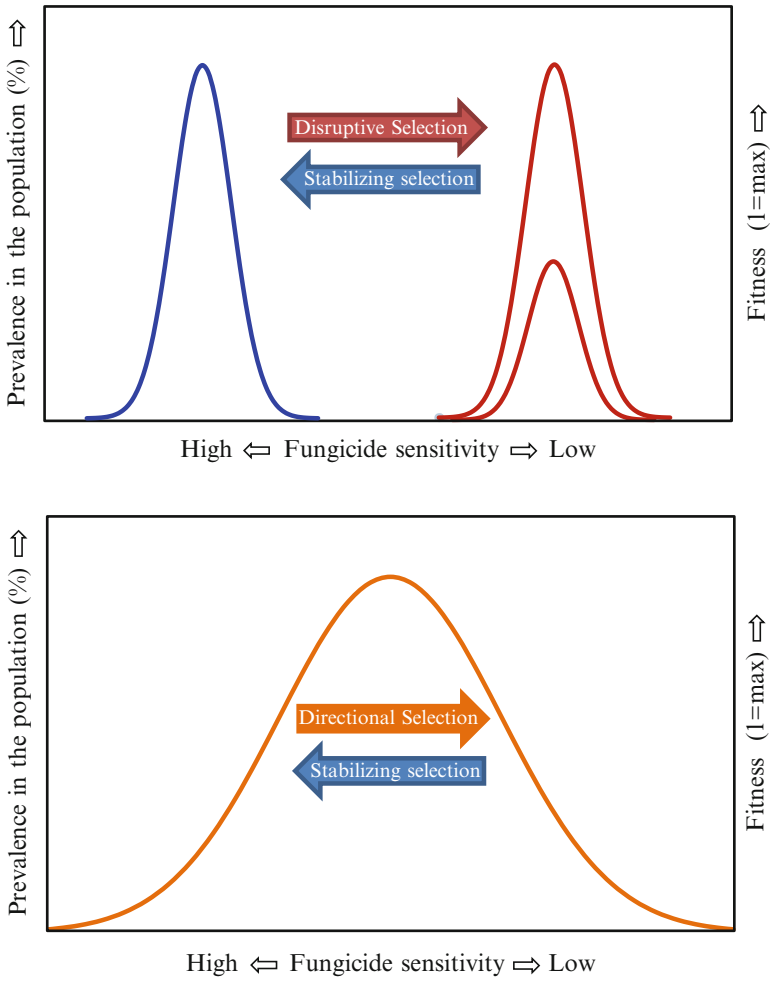

Fig. 2.1 Population dynamics of fungicide resistance in monogenic resistance (*upper*) and polygenic resistance (*bottom*). Disruptive or directional selection is caused by the usage of fungicides having the same mode of action at risk of resistance. Stabilizing selection is due to possible reduction of fitness of fungicide-resistant mutants

in resistance to a same fungicide. For instance, kasugamycin resistance in *Pyricularia oryzae* as well as resistance to the two fungicides ethirimol and triadimenol in *Blumeria graminis* f.sp. *hordei* may be controlled by three different loci where a resistance allele at any one locus confers resistance (Taga et al. 1979; Brown et al. 1992). Furthermore, differently from what is usually observed in most fungi where a single multiallelic gene is responsible for resistance to benzimidazole fungicides, the resistance of *Fusarium oxysporum* to benzimidazoles is caused by mutations in two major genes which interact synergistically conferring high degrees of fungicide resistance (Molnar et al. 1985).

Different mutations in a same gene may cause different levels of resistance to a particular fungicide; this is known as multiallelic resistance. In the past, multiallelic resistance could be assessed only on the ground of phenotypic differences in the level of resistance and/or pleiotropic effects of mutations. With the availability of molecular and sequencing tools, nowadays it is clear that multiallelic resistance is quite common (Table 2.1).

Each mutant allele can be partially/completely dominant or partially/completely recessive to its wild-type allele. That is, when mutant and wild-type alleles of the same gene are combined in the same fungal cells or hyphae, the phenotype may be fungicide resistant (mutant) or fungicide sensitive (wild type).

Combinations of major genes may interact when they are present in the same fungal cells, so that the phenotype of a double mutant may be different from either single-gene mutants (Molnar et al. 1985). Usually, however, one mutant gene is epistatic to another mutant gene, which means that the double mutant has the same level of resistance of the single-gene mutants (Kappas and Georgopoulos 1970; Van Tuyl 1977). The presence of modifier genes affecting phenotypic response of resistant mutants has been suggested to influence the expression of response to phenylamides in *Oomycete* pathogens (Crute and Harrison 1988) or to mediate fitness of resistant mutants as found in mutants of *N. crassa* resistant to dicarboximides (Grindle and Dolderson 1986) and *A. nidulans* resistant to imazalil (van Tuyl 1977). The consequent increase in fitness will result in better survival and possible selection of resistant subpopulations in the field.

Polygenic resistance is due to mutations in minor genes. Those have individually a little effect on the phenotype and cause hence a negligible reduction in the sensitivity to a fungicide. However, numerous mutated minor genes may contribute, with an additive effect, to produce an appreciable increase of the level of resistance. In the field, the result is a quantitative decrease of the sensitivity to a fungicide with a slow, continuous and gradual shift of the fungal population towards increasing resistance levels (Fig. 2.1). Polygenic resistance is much more difficult to be detected and ascertained in the field. Polygenic resistance was demonstrated in *B. graminis* f.sp. *hordei* to ethirimol (Hollomon 1981) and triadimenol (Hollomon et al. 1984). Resistance to dodine is polygenic in *Nectria haematococca* var. *cucurbitae* (Kappas and Georgopoulos 1970). Ultraviolet-induced mutants of *N. haematococca* var. *cucurbitae* also show polygenic inheritance for resistance to fenarimol (Kalamarakis et al. 1991), fenpropimorph and terbinafine (Lasseron-deFalandre et al. 1991).

Cytoplasmic genes are present in mitochondria, plasmids and viruses. Mitochondrial genome, which contains mitochondrial rRNA genes and some of the proteins of the respiratory chain, is the most relevant among fungal extrachromosomal genetic elements affecting resistance to chemicals. However, antibiotic-resistance genes have been located on fungal episomes, plasmids or viruses (Guerineau et al. 1974).

Natural or induced resistance to QoI fungicides, inhibitors of mitochondrial respiration at the Qo site of the cytochrome *bc1* complex (complex III), is usually

conferred by point mutations in the mitochondrial *cytb* gene causing amino acid substitutions in the target protein. In particular, at least three possible codon changes have been associated to a moderate (F129L or G137R) or, more frequently, high (G143A) level of resistance to QoIs in several fungal species (Grasso et al. 2006; Fernández-Ortuño et al. 2008). The presence of a G143-associated group I-like intron in the *cytb* gene in some fungal species (i.e. *Puccinia* spp., *Uromyces appendiculatus*, *Alternaria solani*) or isolates (i.e. *B. cinerea*) prevents the occurrence of the G143A mutation and QoI resistance, since it would be lethal because it would be affecting the correct intron splicing process (Grasso et al. 2006).

Analysis of meiotic progenies of appropriate crosses between sensitive and resistant strains confirmed cytoplasmic (maternal) inheritance of QoI resistance in *B. graminis* (Robinson et al. 2002), *Venturia inaequalis* (Steinfeld et al. 2002) and *B. cinerea* (De Miccolis Angelini et al. 2012a). The segregation pattern in randomly collected progenies is expected to be in a phenotypic 1:0 ratio in most fungal species showing a uniparental, anisogamous inheritance of mitochondrial genome or 1:1 ratio in species, such as *A. nidulans* and *B. graminis* f.sp. *tritici*, showing an hermaphroditic, isogamous mitochondrial inheritance (Robinson et al. 2002).

Wild-type and mutated mitochondrial DNA carrying the G143A mutation in the *cytb* gene may coexist in heteroplasmic state within a single isolate, as demonstrated in several species, including *V. inaequalis* (Zheng et al. 2000), *B. cinerea* (Ishii et al. 2009) and other fungal pathogens (Ishii et al. 2007). Equilibrium between resistant and sensitive mitochondria depends on the strength of selective pressure (Ishii 2010). In *Podosphaera leucotricha*, the relative proportion of mutated and wild-type mitochondria is associated with differences in QoI sensitivity levels of the isolates (Lesemann et al. 2006). An instability of QoI resistance in heteroplasmic isolates grown in absence of selective pressure has been frequently reported (Ishii 2012a) suggesting a fitness cost associated to the resistance (Markoglou et al. 2006).

2.3 Ploidy Level

Differences in ploidy level, affecting the number of alleles at each locus, constitute a major genomic trait influencing the onset and subsequent evolution of fungicide resistance. Firstly, frequency of mutations that may arise in single individuals is directly related to the ploidy level as a result of the different numbers of mutational targets (Otto and Gerstein 2008).

Most phytopathogenic fungi are in haploid state for the major part of their life cycle. In contrast, *Oomycetes* typically show a diploid life cycle and the haploid phase is restricted to the gametes (Fincham et al. 1979). Furthermore, polyploids have been frequently identified among *Oomycetes*, such as *Plasmopara viticola* and *Phytophthora* spp. (Rumbou and Gessler 2006; Bertier et al. 2013).

In haploid fungi, mutations conferring resistance are immediately expressed and then directly exposed to selection, while in diploids or polyploids, mutations first appear in heterozygotic state and their phenotypic effects can be masked by dominant wild-type alleles on the homologous chromosome. For this reason, resistance mutations spread more rapidly in haploid than in diploid or polyploid populations. Fixation time may be reduced and selection against deleterious pleiotropic effects of mutations is more effective in haploids than in diploids (Anderson et al. 2004; Otto and Gerstein 2008).

CAA (carboxylic acid amide) fungicides, inhibitors of cellulose biosynthesis in *Oomycete* phytopathogens, are considered at low to medium resistance risk depending on the fungal species. Resistance to CAAs in *P. viticola* is controlled by one or two recessive nuclear genes, as demonstrated through sexual crosses between CAA-sensitive and CAA-resistant isolates and analysis of segregation patterns of sensitive and resistant phenotypes in F1 and F2 progenies (Gisi et al. 2007; Blum and Gisi 2008) and by sequence analysis of putative resistance genes (Blum et al. 2010). Classic genetic analysis also showed that resistance to all CAA fungicides co-segregates and has thus the same genetic basis (Young et al. 2005; Gisi et al. 2007). However, no cross resistance exists between CAA and other fungicides currently available against *Oomycetes*, such as phenylamides and QoI fungicides, where the intrinsic risk of resistance is estimated to be significantly higher than CAA due to their genetic differences. Resistance to phenylamides is indeed a monogenic trait, conferred by a semidominant chromosomal gene (Gisi and Cohen 1996; Knapova et al. 2002), while QoI resistance is due to mutations in the mitochondrial *cytb* gene (Gisi and Sierotzki 2008).

Similar to CAA, resistance to the new benzamide zoxamide in isolates of *Phytophthora capsici* is recessive and is conferred by two nontarget nuclear genes (Bi et al. 2014). This implies that resistance phenotype is expressed only in homozygous mutants, thus limiting resistance spreading and risk.

Nevertheless, the risk of resistance is significantly increased by the occurrence of gene recombination, even if several cycles of sexual process may be required for making resistance fixed and fully expressed in phenotypically aggressive and well-adapted isolates of the pathogen. Sexual recombination naturally occurring under field conditions has been proposed, for instance, as a possible explanation of the higher risk of CAA resistance assessed in field populations of *Pseudoperonospora cubensis* as compared to in vitro estimations (Zhu et al. 2007). Moreover, CAA resistance has been experienced in *P. viticola* field populations since shortly after their introduction, while no reduced sensitivity to CAA has been detected in other *Oomycetes*, such as the late blight pathogen, *Phytophthora infestans*, despite their intensive usage against these pathogens and extensive monitoring. It has been suggested that the lower risk of CAA resistance in *P. infestans* may be due to the lower frequency of sexual recombination under field conditions, as well as to polyploidy, heterokaryosis (Catal et al. 2010) and chromosomal aberrancies (Gisi 2012).