Chapter 2
Genetics of Fungicide Resistance

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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)

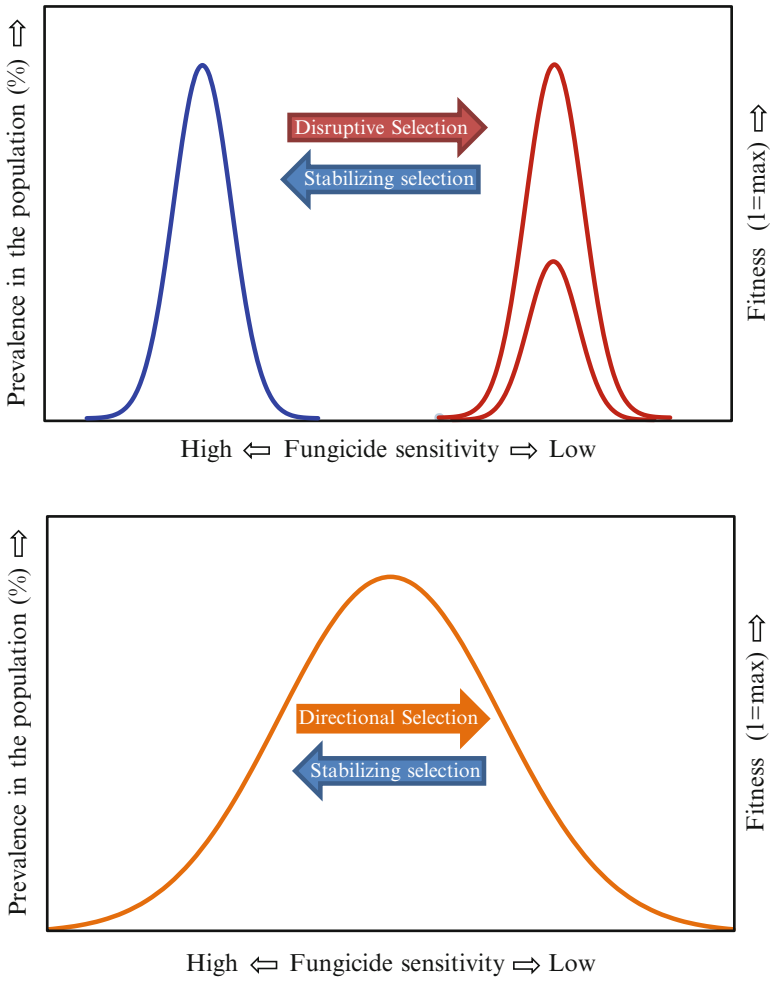

Fig. 2.1 Population dynamics of fungicide resistance in monogenic resistance (*upper*) and polygenic resistance (*bottom*). Disruptive or directional selection is caused by the usage of fungicides having the same mode of action at risk of resistance. Stabilizing selection is due to possible reduction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

2.4 Heterokaryosis and Nuclear Number

The presence of two or more genetically different haploid nuclei, coexisting in a common hyphal compartment, occurs frequently in some fungal *taxa* and is a potential source of genetic variation. In multinucleate *Ascomycetes*, this condition, known as heterokaryosis, often permits changes in the proportions of different nuclei in response to selection and is a prerequisite to parasexual recombination (Davis 1966). In heterothallic *Basidiomycetes*, two distinct parental haploid nuclei coexist without fusion in each cell establishing a stable dikaryotic state. The dikaryon is genetically equivalent to a diploid, as two haploid genomes of different origins exist in each cell even if they remain separated in different nuclei.

Heterokaryons and dikaryons, harbouring several nuclei, offer the opportunity of genes to complement each other (genetic complementation). Heterokaryons harbouring both fungicide-resistant and fungicide-sensitive nuclei may be able to grow in the presence or absence of fungicides (Grindle 1987). They can adapt to fluctuations in fungicide exposure as a result of changes in the proportions and distribution of resistant-sensitive nuclei within cells (Meyer and Parmeter 1968; Ogden and Grindle 1983). Nucleotypic competition and selection after exposure to fungicides and the ability of heterokaryons to adapt to modified environmental conditions have been demonstrated, for instance, in *B. cinerea* strains resistant to dicarboximides (Summers et al. 1984) or to anilinopyrimidines (Santomauro et al. 2000).

Dominance or recessivity can be tested by inducing hyphal anastomosis between one strain carrying the wild-type allele and the other the mutant allele of a resistance gene to form heterokaryotic mycelium. Incompatibility impeding heterokaryon establishment can be overcome by fusion of protoplasts. The mutant allele is completely (or partially) dominant if the heterokaryon is phenotypically identical (or similar) to the mutant parent; it is completely (partially) recessive if the heterokaryon is phenotypically similar to the wild-type parent (Grindle 1987; Grindle and Faretra 1993).

2.5 Level of Resistance and Pleiotropic Effects of Resistance Mutations

Levels of resistance are usually quantified by determining from dose–response curves the concentration of fungicide needed to reduce 'life' parameters, such as colony or mycelium growth or spore germination by 50 % (effective concentration 50; EC_{50}) and the minimal inhibitory concentration (MIC). A mutant can be designated resistant to a fungicide if its EC_{50} value is at least twice the EC_{50} value of sensitive wild-type isolates (Delp and Dekker 1985). However, small differences in EC_{50} values among resistant mutants and sensitive isolates may not be detected

unless environmental variables are rigorously controlled and/or data from dose–response experiments are subjected to statistical analysis. Moreover, with small differences it may be difficult to establish whether resistance is due to a single major gene or to polygenes (Grindle and Faretra 1993).

A mutant gene conferring resistance to a particular fungicide often confers positive cross resistance to other fungicides having the same or related mode of action. On the contrary, mutant genes causing resistance to one fungicide may increase sensitivity to other chemicals (negative cross resistance) (Brent and Hollomon 2007a, b).

Resistance mutations may have deleterious pleiotropic effects on unrelated phenotypic characters, such as competitiveness, virulence, survival and reproductive success. Physiological mechanisms underlying resistance to fungicides may also be associated with a metabolic cost. Hence, resistant isolates may have lower fitness than wild-type sensitive isolates. Differences in fitness can be experimentally measured as reduction in mycelial growth rate, sporulation and conidial germination, pathogenicity, survival under stressing conditions, etc., in a fungicide-free environment. For instance, fitness penalty was observed in (1) DMI resistance in powdery mildews (Gisi et al. 2002); (2) resistance to dicarboximides and phenylpyrroles in several fungi, such as *B. cinerea* (Pollastro et al. 1996; Ochiai et al. 2001), *N. crassa* (Hollomon et al. 1997) and *Monilinia laxa* (Katan and Shabi 1982); (3) resistance to QoIs (G143A replacement) in field populations of *P. viticola* (Fernández-Ortuño et al. 2008) and *Pyricularia grisea* (Avila-Adame and Köller 2003) and in laboratory mutants of *B. cinerea* (Markoglou et al. 2006), *C. beticola* (Malandrakis et al. 2006) and *Ustilago maydis* (Ziogas et al. 2002), but not in *B. graminis* f.sp. *tritici* (Heaney et al. 2000; Chin et al. 2001), *Mycosphaerella graminicola* (Miguez et al. 2004) and *Magnaporthe grisea* (Avila-Adame and Köller 2003); (4) most of the mutations in the *SdhB* gene conferring resistance to SDHIs in *B. cinerea* except for $SdhB^{H272Y}$ (Lalève et al. 2014a; Veloukas et al. 2014); and (5) *Penicillium expansum* resistant to tebuconazole, fludioxonil and iprodione, but not to cyprodinil, coupled with reduction in patulin production (Karaoglanidis et al. 2011).

Mutations responsible for fungicide resistance may influence mycotoxin production. For instance, the production of 3-acetyl deoxynivalenol (3-ADON) was altered in isolates of *Fusarium culmorum* resistant to the DMI fungicide difenoconazole (D'Mello et al. 1997). A higher production of T-2 toxin, 4,15-diacetoxyscirpenol and neosolaniol was found in a carbendazim-resistant strain of *Fusarium sporotrichioides* (D'Mello et al. 1998, 2000). More recently, Zhang et al. (2009) found that benzimidazole resistance increased trichothecene production in *F. graminearum*. Laboratory mutants of *Aspergillus parasiticus* resistant to phenylpyrroles and dicarboximides produced more aflatoxins than the parental wild-type strain (Markoglou et al. 2008a). Similarly, laboratory mutant strains of *A. parasiticus*, *A. ochraceus* and *F. verticillioides* resistant to triazoles (epoxiconazole and flusilazole) and mutant strains of *A. carbonarius* and *P. expansum* resistant to fludioxonil produced significantly higher levels of mycotoxins (ochratoxins, patulin and fumonisins) compared to the parental sensitive strains (Doukas et al. 2008; Markoglou et al. 2008b, 2009).

It is generally assumed that fitness costs of resistance are invariable. However, Chin et al. (2001) showed that the cost of resistance to QoI fungicides in *B. graminis* varies with environmental conditions, such as temperature, being more costly under suboptimal conditions for the fungus.

2.6 Population Genetics

To develop effective resistance management strategies, it is crucial to know all the factors influencing relationship between sensitive and resistant strains.

The prevailing model explaining the selection of fungicide-resistant fungal populations considers random and rare mutations as the cause for pre-existing but infrequent resistant phenotypes prior to the introduction of a new fungicide (Torriani et al. 2009; Camps et al. 2012). Nevertheless, the evolutionary question on how populations adapt to novel environments, such as new antimicrobials, through de novo mutations or through selection from standing genetic variation, which affect the probability and speed of emergence of resistant alleles, is still debated (Hermisson and Pennings 2005; Hawkins et al. 2014).

Anyway, rare resistant mutants gain in competitiveness under the selection force of fungicide sprays and are selected to frequencies at which disease control becomes unsatisfactory (Milgroom et al. 1989; Skylakakis 1987; Wolfe 1982; Hobbelen et al. 2014). The shift towards resistance occurs at different rates depending on the number of genes conferring resistance. In monogenic resistance, a rapid shift towards resistance may occur, leading to discrete resistant subpopulation(s), while in polygenic resistance, the shift towards resistance progresses slowly, leading to a reduced sensitivity of the entire population. Resistant and wild-type subpopulations are in a dynamic equilibrium due to two selective pressures: i) the disruptive selection (directional selection in polygenic resistance), favouring resistant subpopulation(s), is due to repeated sprays with fungicides having the same mode of action at risk of resistance, and ii) stabilizing selection, favouring the wild-type sensitive populations, is caused by possible negative pleiotropic effect of resistance mutations leading to a reduced fitness (Fig. 2.1). Unfit mutants compete well only under the selection pressure of fungicide sprays, and, hence, resistance is at least partially reversible when the selection pressure is removed or minimized by applying resistance management strategies.

2.7 Obtainment of Resistant Mutants

Field isolates collected from diseased plants, plant debris, soil or air may include fungicide-resistant mutants, particularly if crops have been sprayed intensively with single-site fungicides. Resistant field isolates may be selected on appropriate agar

media amended with a fungicide at a concentration inhibiting germination of conidia and/or mycelium growth of wild-type sensitive isolates. In choosing agar medium the mode of action of the fungicide must be complained. In the case of obligate biotrophic pathogens, plants or parts of plants must replace agar media. A number of monitoring methods are available (www.frac.info). Field isolates may display a broad variation making their genetic analysis more difficult than laboratory mutants (Grindle and Faretra 1993). It is advisable to obtain 'monoconidial' or 'single hyphal tip' isolates rather than 'mass-conidial' or 'mass-hyphal' isolates since they are likely to be genetically more homogeneous and stable. These traits are improved by repeated subculturing monoconidial isolates selecting the 'most typical' progeny.

Experiments under laboratory conditions are useful because it is possible to replicate them, to control the strength of selection and to use defined reference strains (Cowen et al. 2002). Resistant laboratory mutants can be generated in vitro from wild-type strains of known phenotype, and all mutants deriving from a same strain are near isogenic since their genomes are virtually identical, except for mutant gene(s) conferring resistance.

Selection of spontaneous mutations may be achieved by growing fungal colonies on media added with sublethal fungicide concentration; resistant hyphae grow better than the sensitive ones and produce vigorous sectors from slow-growing colonies. Alternatively, a high number of conidia can be plated on fungicide-amended media, and growing colonies can be singly transferred to fresh media. For instance, in *B. cinerea* it is relatively easy to get spontaneous mutants resistant to dicarboximides, phenylpyrroles, anilinopyrimidines and QoIs (Faretra and Pollastro 1991, 1993a, b; De Miccolis Angelini et al. 2002, 2012a).

Mutations can be induced by exposing conidia or hyphae to chemical (e.g. N-methyl-N-nitro-*n*-nitrosoguanidine) or physical mutagens (e.g. UV light) causing established proportions of lethality, before incubation on selective media. Mutagenesis greatly increases the yield of mutants but may cause unwished mutations in the genome which may interfere with identification and analysis of gene(s) causing fungicide resistance. Physical or chemical mutagenesis have been used successfully to produce resistant mutants in numerous fungi, including *B. cinerea* (De Miccolis Angelini et al. 2002, 2010a, b, 2012a, b), *F. graminearum* NRRL 13383 (Becher et al. 2010), *P. capsici* and *P. infestans* (Young et al. 2001), *Ustilago maydis* (Orth et al. 1994) and *V. inaequalis* (Zheng et al. 2000). In fungi with multinucleate conidia, laboratory mutants are frequently heterokaryons containing both mutated and wild-type nuclei so that they are often phenotypically unstable and produce both resistant and wild-type progeny during subculturing. Hence, at least initially, the selective pressure exerted by fungicide is crucial for the stability of the resistance trait.

The availability of molecular techniques has made it possible to investigate the genomes of pathogenic fungi which are not amenable to classical Mendelian analysis of meiotic progeny. For instance, genetic differences between isolates can be detected by RFLP (restriction fragment length polymorphisms) or various PCR-based techniques suitable for evidencing SNPs (single nucleotide polymorphisms)

and allelic variants (AS-PCR, allele-specific PCR). Individual genes can be dissected out of the genome, then cloned and sequenced or altered genetically and put back into the genome. Cells containing cloned genes can be used to obtain large amounts of protein for amino acid sequencing. In the last fifteen years, site-direct mutagenesis has been used for studies of fungicide resistance. For instance, *N. crassa* mutants in the osmosensing histidine kinase *os-1* gene exhibit resistance to dicarboximides, aromatic hydrocarbons and phenylpyrroles. The *os-1* mutants can be classified into two groups: type I are null mutants highly resistant to iprodione and fludioxonil and moderately sensitive to osmotic stress, and type II carry single amino acid changes and are moderately resistant to both fungicides and highly sensitive to osmotic stress. This suggests that Os1p is essential for the antifungal activity of these fungicides and that amino acid repeats have an important function in osmoregulation (Ochiai et al. 2001). Site-directed mutagenesis followed by gene replacement was used to introduce mutations in different codons of the β-tubulin gene of a carbendazim-sensitive field strain of *Gibberella zeae*. All the mutants were resistant to carbendazim, but the level of resistance was depending on the mutations (Qiu et al. 2011). Site-directed mutagenesis of the *SdhB* gene was applied to confirm that each of the mutations identified in field strains conferred resistance to boscalid in *B. cinerea* and partial cross resistance to other SDHIs (fluopyram, carboxin) (Lalève et al. 2014b).

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