Chapter 14
Sterol Biosynthesis Inhibitors: C-4 Demethylation

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Abstract Two available fungicides block sterol C-4 demethylation in ergosterol biosynthesis: the hydroxyanilide fenhexamid and the amino-pyrazolinone fenpyrazamine, introduced in the early 2000s and 2010s, respectively. Both these fungicides specifically target 3-ketoreductase (Erg27), the third enzyme of the C-4 demethylation enzyme complex. Highly effective against *Botrytis cinerea*, causing gray mold, they are also effective against other pathogens, such as *Monilinia* spp., *Sclerotinia* spp., and *Oculimacula* spp. Up to now, most available data on resistance phenomena have been recorded with fenhexamid on gray mold. The mechanism of acquired resistance to fenhexamid is linked to target modification in most resistant strains. Amino-acid changes due to *erg27* gene mutations possibly decrease the affinity of fenhexamid for 3-ketoreductase. Twenty-three individual mutations, sometimes, but rarely, occurring in pairs, have been identified in moderately resistant strains. The principal highly resistant strains display one out of four detected amino-acid substitutions at position 412. In fungal species naturally resistant to fenhexamid, this intrinsic resistance also appears to be related to a low affinity of the hydroxyanilide for the 3-ketoreductase. Gray mold has recently been shown to be caused by a complex of two cryptic species living in sympatry: *B. cinerea*, the predominant species, and *Botrytis pseudocinerea. B. pseudocinerea* is naturally resistant to fenhexamid, due to its cytochrome P450 protein Cyp684 rather than *erg27* polymorphism. The function of Cyp684 is unknown, but it may be involved in fenhexamid detoxification. Moreover, multidrug-resistant strains exhibiting reduced susceptibility to fenhexamid have been detected in *B. cinerea* and *Oculimacula yallundae*.

Keywords *Botrytis cinerea* • *Botrytis pseudocinerea* • Gray mold • Fungicide resistance • Resistance mechanisms • Fenhexamid • Sterol 3-ketoreductase • *erg27* mutation • Cyp684

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14.1 Introduction

Sterol biosynthesis inhibitors (SBIs) have been used over the last 40 years, in both medicine and agriculture, to control fungal diseases. Ergosterol, the major final sterol in many fungi, is essential for both bulk membrane and metabolic functions. Inhibition of the enzymes involved in ergosterol biosynthesis leads to ergosterol depletion and the accumulation of precursor or abnormal sterols, resulting in fungitoxicity due to alterations of the membrane and metabolic functions (Köller 1992; Debieu et al. 1998, 2001; Akins 2005). The available SBIs can be distributed into four classes on the basis of the enzyme targeted (Fig. 14.1), a membrane-bound enzyme of the endoplasmic reticulum (Leroux et al. 2008). Five of the 15 post-squalene steps in fungal sterol biosynthesis are targeted by these fungicides. Squalene epoxidase (Erg1) is inhibited by allylamines (e.g., terbinafine) and thiocarbamates (e.g., tolnaftate, pyributicarb), which are used mostly in medicine. The 14α-demethylase cytochrome P450 monooxygenase (Erg11 or Cyp51) is the target of commercially important azole drugs (e.g., fluconazole, ketoconazole, posaconazole) and polyvalent DMI (14α-demethylation inhibitor) fungicides (e.g., prochloraz, tebuconazole, epoxiconazole, prothioconazole). The Δ^{14}-reductase (Erg24) and $\Delta^8 \rightarrow \Delta^7$-isomerase (Erg2) are inhibited by tertiary amines (e.g., amorolfine, fenpropimorph, tridemorph, fenpropidin, spiroxamine). These two enzymes may be differently inhibited in different fungi and with different compounds (Debieu et al. 1998, 2000). Amines are mostly used to combat powdery mildews in agriculture. The third enzyme of the sterol C-4 demethylation complex,

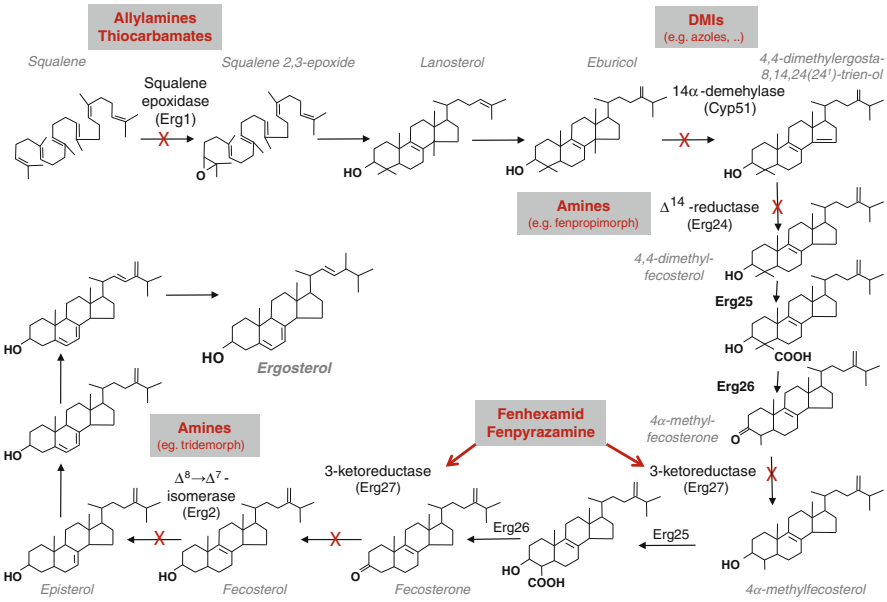
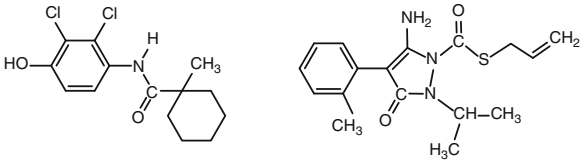


Fig. 14.1 Sterol biosynthesis inhibitors used to control fungal diseases

3-ketoreductase (Erg27), is inhibited by the more recently developed fungicides. This class of fungicides was established in the 2000s, following the elucidation in our laboratory of the mode of action of fenhexamid (Debieu et al. 2001), which was developed by Bayer in 1999 (Krüger et al. 1999). Fenhexamid (1-methyl-cyclohexanecarboxylic acid (2,3-dichloro-4-hydroxy-phenyl)-amide), a hydroxy-anilide derivative, was the only member of this new class of SBIs targeting the sterol 3-ketoreductase until 2012, when Sumitomo Chemical Co. Ltd developed a novel fungicide, fenpyrazamine (S-allyl 5-amino-2,3-dihydro-2-isopropyl-3-oxo-4-(o-tolyl)pyrazole-1-carbothioate), an amino-pyrazolinone derivative targeting the sterol 3-ketoreductase (Tanaka et al. 2012, 2013) (Fig. 14.2). Sterol C-4 demethylation in fungi involves three enzymes (Mercer 1984). A C-4 methyloxidase (Erg25) reaction first converts the 4α-methyl group into an alcohol, then an aldehyde, and, finally, a carboxylic acid (Bard et al. 1996). A C-3 sterol dehydrogenase (Erg26) then removes the 3α-hydrogen, leading to decarboxylation of the 3-ketocarboxylic acid sterol intermediate (Gachotte et al. 1998). Finally, the 3-ketoreductase (Erg27) converts the 3-keto group into a 3β-hydroxyl group (Gachotte et al. 1999). Two consecutive rounds of C-4 demethylation are required to eliminate the two methyl groups on C-4 (Fig. 14.1). In addition to the three enzymes, a protein encoded by *erg28* is thought to facilitate protein-protein interactions within the C-4 demethylation enzyme complex (Gachotte et al. 2001; Mo and Bard 2005). Fenhexamid and fenpyrazamine have a narrow spectrum of activity. They are highly effective against the causal agent of gray mold, *Botrytis cinerea* (Rosslenbroich and Stuebler 2000; Tanaka et al. 2012).

The intensive use of SBIs in medicine and agriculture is leading to the selection of fungal strains with various degrees of resistance to these fungicides. This acquired resistance is often determined by qualitative changes in the target enzymes, possibly resulting in a decrease in the affinity of inhibitors for these proteins (Leroux et al. 2008). Following treatment with azole drugs and DMI fungicides, overexpression of the target *cyp51* gene or of genes encoding membrane transporters increasing the efflux of inhibitors has often been reported (Akins 2005; Cools et al. 2013). This chapter devoted to the sterol 3-ketoreductase inhibitors will review current knowledge about the mechanisms of acquired and natural (intrinsic) resistance to this class of SBIs. Most of the data considered were obtained with fenhexamid.



Fenhexamid
(hydroxyanilide)

Fenpyrazamine
(amino-pyrazolinone)

Fig. 14.2 Chemical structures of fenhexamid and fenpyrazamine

14.2 Is the Narrow Spectrum of Fenhexamid and Fenpyrazamine Activity Related to Their Affinity for the 3-Ketoreductase (3-KR)?

The two available fungicides for blocking sterol C-4 demethylation, fenhexamid and fenpyrazamine, are highly toxic to various *Botrytis* species but especially to *B. cinerea* and the related pathogens *Monilinia* spp. and *Sclerotinia* spp. The two species responsible for cereal eyespot, *Oculimacula acuformis* and *O. yallundae,* are also susceptible to these SBIs. By contrast, these fungicides display little or no activity against many other eumycetes, including powdery mildews, rusts, smuts, *Alternaria* spp., *Fusarium* spp., *Mycosphaerella* spp., *Pyrenophora* spp., *Pyricularia* spp., *Rhizoctonia* spp., *Trichoderma* spp., and yeasts (Rosslenbroich 1999; Tanaka et al. 2012; Debieu et al. 2013; Leroux et al. 2013).

In vitro studies on susceptible species at various stages of development have indicated that these fungicides do not affect spore germination; instead, low concentrations of these compounds inhibit germ tube elongation and mycelial growth (Table 14.1). Furthermore, the alterations to germ tube morphology observed are similar to those recorded with other classes of SBIs (Leroux et al. 1999, 2013; Tanaka et al. 2013). The sterol profile in the mycelia of susceptible species (e.g., *B. cinerea* and *Oculimacula* spp.) treated with fenhexamid is characterized by a decrease in ergosterol content and the production of sterones (steryl compounds with a 3-keto rather than a 3β-hydroxyl function) in large quantities (Debieu et al. 2001 and unpublished, Tanaka et al. 2012). Sterones are fungitoxic (Debieu et al. 2001), particularly because the 3β-hydroxyl group is essential for membrane function and cannot therefore be replaced with a 3-keto group (Nes et al. 1993). The three major sterones are 4α-methylfecosterone, fecosterone, and episterone (derived from fecosterone $\Delta^8 \rightarrow \Delta^7$-isomerization), consistent with inhibition of the 3-ketoreductase (Erg27; 3-KR) (Fig. 14.1). The low concentrations of fungicide required to halve the activity of the *B. cinerea* 3-KR (I_{50}) in microsomal assays indicate that fenhexamid and fenpyrazamine are good inhibitors of this enzyme (Debieu et al. 2007, 2013; Tanaka et al. 2013). By contrast, the sterol profile observed in the fenhexamid-treated mycelia of intrinsically resistant fungi (e.g., *Nectria haematococca*, *Pyrenophora teres*) is similar to that of the control, suggesting that the 3-KR enzyme of these species cannot be inhibited by fenhexamid. For fenhexamid, the I_{50} value for *N. haematococca* 3-KR is about 200 times higher than that for *B. cinerea* (Debieu et al. 2013). Thus, intrinsic resistance to this fungicide seems related to a low affinity for the target site. It could therefore be hypothesized that the narrow activity spectra of fenhexamid and, probably, fenpyrazamine reflect their affinity for the 3-KR.

Most fungal NADPH-dependent 3-KR enzymes are characterized by two common features of short-chain dehydrogenase/reductase enzymes: an active catalytic site with the consensus sequence YXXXK and a terminal coenzyme binding site with a characteristic GXXXGXG motif (GANSGI/LG in filamentous fungi but with

Table 14.1 Resistance to the hydroxyanilide fenhexamid in field strains of the causal agents of gray mold

Characteristics	*Botrytis cinerea*					*Botrytis pseudo-cinerea*[c] (HydR1)
	Wild-type	HydR2[a]	HydR3$^-$	HydR3$^+$	MDR[b]	
In vitro response[d] *to fenhexamid:*						
GT elongation	0.04[e]	S[f]	MR	HR	LR	S
Myc growth	0.016	MR	MR	HR	S	HR
In vitro response[d] *to edifenphos*[g]:						
Myc growth	R	R	R	R	R	S
Strong synergism:						
DMI fungicides	No	Yes	No	No	No	Yes
Resistance basis	–	Detoxification: un-known P450	*Erg27* mutations: reduced affinity of fenhexamid to 3-KR		Efflux pump *BcmfsM2* overproduction	Detoxification: Cyp684(P450)

[a]HydR2, HydR3$^-$ HydR3$^+$ phenotypes resistant to the hydroxyanilide fenhexamid (Leroux et al. 2002a; Fillinger et al. 2008)
[b]MDR: multidrug-resistant strains, MDR2 and MDR3 phenotypes (Kretschmer et al. 2009; Leroux and Walker 2013)
[c]New species, previously referred to as the HydR1 phenotype, naturally resistant to fenhexamid (Walker et al. 2011)
[d]GT elongation: germ-tube elongation; Myc growth: mycelial growth
[e]EC_{50} value expressed in mg.1^{-1} for fenhexamid in wild-type strains
[f]Levels of resistance to fenhexamid estimated as the ratio EC_{50} for the resistant phenotype/EC_{50} for wild-type strains, below 3= S (sensitive); 3–10 = LR (low resistance); 10–100 = MR (moderate resistance) or greater than 100 = HR (high resistance)
[g]EC_{50} values for edifenphos: below 1 mg.1^{-1} = S or higher than 10 mg.1^{-1} = R

N instead of the second G in yeasts) (Oppermann et al. 2003). Moreover, species susceptible to fenhexamid (e.g., *B. cinerea*, *S sclerotiorum*) have a longer Erg27 sequence than fungal species intrinsically resistant to this fungicide. The two largest extensions are located in the N-terminal part of the molecule, on either side of the NAGI motif, the function of which remains unknown. Finally, the presence of a putative transmembrane domain (TMD) with helical structure at the C-terminal end of the protein is not observed in all fungal species (Fig. 14.3) (Albertini and Leroux 2004; Fillinger et al. 2008; Billard et al. 2011; Debieu et al. 2013). Three-dimensional molecular homology modeling of the fungal 3-KR will provide insight into the differences in susceptibility between species resulting in the narrow spectrum of activity of fenhexamid and fenpyrazamine.

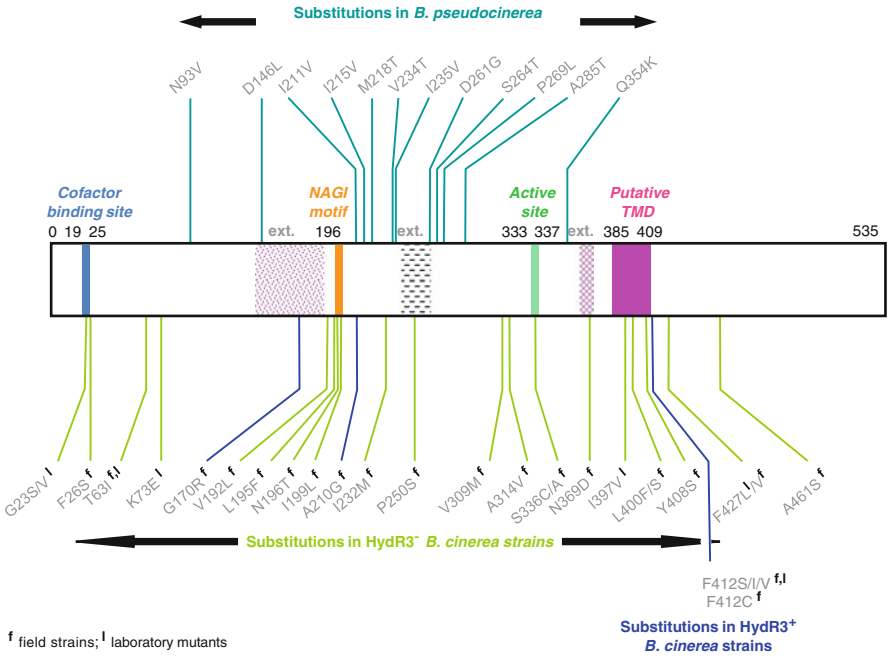


Fig. 14.3 Schematic representation of Erg27 polymorphism in *Botrytis cinerea* and *Botrytis pseudocinerea* (Adapted from Billard et al. 2011). The 12 amino acids that differ between Erg27 *B. pseudocinerea* and *B. cinerea* are indicated above the sequence; the amino-acid substitutions resulting from *erg27* mutations in *B. cinerea* fenhexamid-resistant strains that have been published to date are shown below the sequence. The cofactor (NADPH) binding site, NAGI motif (unknown function), active site, putative transmembrane domain (TMD), and the three main extensions (ext) of the peptide sequence relative to other resistant fungal species (Debieu et al. 2013, supporting information) are indicated in *boxes*

14.3 *Botrytis pseudocinerea* Is a New Species Naturally Resistant to Fenhexamid

Before the release of fenhexamid onto the fungicide market, strains intrinsically resistant to this hydroxyanilide were detected within field populations of *B. cinerea* in Europe (Leroux et al. 1999; Suty et al. 1999). These strains, in which germ tube elongation was sensitive to fenhexamid, but mycelial growth was highly resistant to this fungicide, were referred to as phenotype HydR1 strains (Leroux et al. 2002a, b, 2004). These HydR1 strains with natural resistance to fenhexamid actually correspond to the recently described species *Botrytis pseudocinerea* causing gray mold in sympatry with *B. cinerea* (Walker et al. 2011). These two cryptic species are morphologically indistinguishable, but they display genetic polymorphism for several of the nuclear genes studied, including *erg27* in particular (Albertini and Leroux 2004; Walker et al. 2011). The Erg27 protein sequence of *B. pseudocinerea* differs from that of *B. cinerea* by 12 amino-acid residues (i.e., N93V, D146N, I211V,

I215L, M218T, V234A, I235V, D261G, S264T, P269L, A285T, and Q354K) (Albertini and Leroux 2004). These 12 different amino-acid residues (Fig. 14.3), distributed along the length of the protein, are not located at the NADPH binding site, the NAGI motif, the active site, or the predicted transmembrane domain (TMD) (Albertini and Leroux 2004). In microsomal assays, a higher concentration of fenhexamid was required to inhibit the 3-KR of *B. pseudocinerea* than that of *B. cinerea* (Debieu et al. 2013). Moreover, it has been shown that *erg27* is not overexpressed in *B. pseudocinerea* (Billard 2011), suggesting a decrease of affinity of fenhexamid for *B. pseudocinerea* 3-KR. However, *B. cinerea* transformants harboring the *B. pseudocinerea erg27* gene exhibit a low resistance to fenhexamid, indicating that the *B. pseudocinerea erg27* polymorphism is not the major player in the natural resistance observed (Billard 2011; Billard et al. 2011). Thus, the natural resistance of *B. pseudocinerea*, expressed at mycelial growth stage, appears to be slightly related to the enzyme targeted by fenhexamid, Erg27, a supplemental resistance mechanism that has to be found.

Suty et al. (1999) showed that *B. pseudocinerea* strains metabolized fenhexamid, suggesting a possible resistance mechanism unlinked to the target of the fungicide. Moreover, the strong synergism observed between fenhexamid and DMI fungicides in *B. pseudocinerea* strains but not in *B. cinerea* strains suggests the possible involvement of a cytochrome P450 monooxygenase in fenhexamid detoxification (Leroux et al. 2000, 2002a). This detoxification possibly involved hydroxylation at various positions in the cyclohexyl ring (Leroux et al. 2002b). Among the available *B. cinerea* genome database (Amselem et al. 2011) (http://urgi.versailles.inra.fr/species/Botrytis), 60 putative cytochrome-encoding genes similar to *B. cinerea cyp51* were identified by blastp. Only one of these genes was found to be overexpressed in fenhexamid-treated *B. pseudocinerea* mycelia and not in *B. cinerea* (Billard et al. 2011; Azeddine 2014). According to the nomenclature of P450s, this *Bc1G_02902* gene was named *cyp684*. Its inactivation in *B. pseudocinerea* has been shown to lead to the loss of both natural fenhexamid resistance and synergism between fenhexamid and DMI fungicides (Billard 2011; Billard et al. 2011; Azeddine 2014). Moreover, *B. pseudocinerea* can also be distinguished from *B. cinerea* biologically on the basis of its susceptibility to edifenphos (Table 14.1) (Leroux et al. 2000, 2002a), a phosphorothiolate used against *P. oryzae*. This susceptibility is lost following *cyp684* inactivation (Billard 2011; Billard et al. 2011). Edifenphos is a profungicide, yielding unstable intermediate metabolites (i.e., phenylmercaptan or oxidized derivatives) on P-S cleavage that inhibit fungal phospholipid biosynthesis (Uesugi and Takenaka 1993; Sugiura et al. 1993). These results suggest that Cyp684 plays a major role in detoxifying fenhexamid and activating edifenphos in *B. pseudocinerea*.

In *B. cinerea* and *B. pseudocinerea*, *cyp684* encodes a 500-amino-acid protein with a sequence typical of eukaryotic P450 class II (Werck-Reichhart and Feyereisen 2000). The best protein blast hits are other fungal P450s from species for which complete genome sequences have been published, such as *Aspergillus nidulans*, *Fusarium oxysporum*, and *N. haematococca*. However, the functions of these proteins are unknown. Surprisingly, the *cyp684* gene is absent from *S. sclerotiorum,*

which is phylogenetically closer to *B. cinerea* and *B. pseudocinerea* than fungal species listed above. The *Botrytis* spp. Cyp684 sequence comprises three characteristic P450 domains: (1) the heme-binding site including the sequence FGGGSRVCLG (consensus FXXGXRXCXG) with a cysteine residue acting as a fifth ligand for heme iron; (2) the absolutely conserved EXXR motif, which takes the form ETLR in *Botrytis* spp. This motif is located in helix K, close to the heme, and is probably required to stabilize the core structure and (3) the AGSDTT sequence (consensus A/G GX D/E T T/S) of the central part of helix I, involved in proton transfer to heme. Moreover, *Botrytis* spp. Cyp684 contains a membrane-targeting hydrophobic domain at its N-terminal end, mediating anchorage to the endoplasmic reticulum (Werck-Reichhart and Feyereisen 2000; Billard 2011; Azeddine et al. 2012; Azeddine 2014). A comparison of Cyp684 proteins from *B. cinerea* and *B. pseudocinerea* revealed the presence of four polymorphic amino acids, two of which were located in the TMD (V30I and I39T) and the other two being located in the β helix (A100T) or the loop between the F and G helices (for I236M) (Azeddine et al. 2012; Azeddine 2014). This loop is generally thought to be located within the substrate access channel of eukaryotic P450s (Werck-Reichhart and Feyereisen 2000). Moreover, in *B. pseudocinerea cyp684,* a 25 bp deletion in the promoter region and a 24 bp insertion in the 3′UTR may be responsible for the overexpression of *cyp684* in *B. pseudocinerea* (Billard 2011; Azeddine 2014). Further studies are required to assess the impact of *cyp684* mutations and overexpression on the natural resistance of *B. pseudocinerea* and their relative importance for fungicide metabolization.

14.4 Acquired Resistance to Fenhexamid in *Botrytis cinerea*

Up to now, most available data on acquired resistance mechanisms to fenhexamid have been recorded with *B. cinerea*. Two types of fenhexamid resistance phenotype were detected in *B. cinerea* populations, on the basis of the in vitro effects of this hydroxyanilide. These phenotypes were named HydR2 and HydR3 by Leroux et al. (2002a, b). In the small number of HydR2 strains identified to date, resistance is expressed principally at the mycelial growth stage. By contrast, in the more frequent HydR3 strains, resistance is expressed at both the germ tube elongation and mycelial growth stages (Table 14.1).

The first HydR2 strains were collected in the late 1990s, in trials conducted by Bayer on various crops, in Japan and Germany. Their in vitro response to fenhexamid resembles that of *B. pseudocinerea,* resistance but with a lower level of resistance. These HydR2 strains rarely found are not detected in French vineyards (Walker et al. 2013) but possibly, at low frequency, on table grape and strawberry in Southern Italy (De Miccolis Angelini et al. 2014). Recently, Amiri and Peres (2014) detected isolates with reduced sensitivity from strawberry fields in Florida; they published them as HydR2 phenotype, according to their fenhexamid sensitivity evaluation. These isolates are surprisingly representing 25 % of the Florida population. Their frequencies are higher than that of HydR3^{-} strains but are controlled in

fields. More investigations are required to determine both the phenotype and genotype of these isolates for their identification in monitoring of gray mold populations. With the strains isolated from trials conducted by Bayer, strong synergism was also noted between this hydroxyanilide and DMI fungicides (Leroux et al. 2002a, b). The absence of expressed mutations in the *erg27* gene of HydR2 strains (Albertini and Leroux 2004; Billard 2011) suggests that the principal mechanism of resistance may be the detoxification of fenhexamid by a cytochrome P450 monooxygenase, other than Cyp684, that cannot activate edifenphos (Table 14.1) (Billard 2011). Some HydR2 strains produce the red polyketide pigment bikaverin. The production of this pigment is due to the presence of a gene cluster probably acquired from *Fusarium* spp. by horizontal gene transfer. The HydR2 gene locus is genetically linked to this cluster, but none of its six genes seem to be involved in fenhexamid resistance. Moreover, the expression of both the genes of this cluster and the HydR2 genes is upregulated by Bc VEL1, a protein of the VELVET complex that has been reported to be a global regulator of secondary metabolism and differentiation in fungi (Schumacher et al. 2012, 2013).

HydR3 strains display moderate to high levels of resistance to fenhexamid and, possibly, fenpyrazamine, at both the germ tube elongation and mycelial growth stages (Table 14.1) (Fillinger et al. 2008; Tanaka et al. 2012). This phenotype, which has been found in laboratory mutants and, since 2003, in field strains collected from commercial crops, is associated with mutations of the *erg27* gene resulting in changes to the 3-KR protein. In field strains moderately resistant to fenhexamid ($HydR3^-$ strains), the following individual amino-acid changes have been found, sometimes (but rarely) in pairs: F26S, T63I, V192L, L195F, I 199 L, N196T, I232M, P250S, V309M, A314V, S336C/A, N369D, L400F/S, Y408S, F427V, and A461S (Fig. 14.3) (Fillinger et al. 2008; Esterio et al. 2011; Grabke et al. 2013; Amiri and Peres 2014; Saito et al. 2014). In laboratory, mutants with a similar phenotype, T63I substitution has also been found, but others such as G23S/V, K73E, I397V, and F427L substitutions have been observed (De Miccolis Angelini et al. 2012; Saito et al. 2010). Highly resistant strains ($HydR3^+$ strains), with an individual mutation in *erg27*, causing an amino-acid substitution at position 412, have been found in both field strains and laboratory mutants. The most frequent change is the replacement of a phenylalanine with a serine or, more rarely, an isoleucine or valine residue (Fillinger et al. 2008; Saito et al. 2010; Esterio et al. 2011; De Miccolis Angelini et al. 2012). In the Carolinas strawberry fields, in addition to F412S or F412I changes found in resistant strains, Grabke et al. (2013) recorded for the first time the replacement of a phenylalanine with a cysteine residue. Recently, Amiri and Peres (2014) have detected, in addition to strains bearing F412 mutation, strains exhibiting two single mutations, not yet recorded, leading to an amino-acid substitution at position 170 or 210. The resistance level of these strains, found at low frequency, seems to be intermediate between moderate and high according to resistance factor scale for $HydR3^-$ and $HydR3^+$ phenotypes. For these strains, it would be interesting to make in vitro test at both germ tube elongation and mycelial growth stages to confirm $HydR3^+$ phenotype, as initially characterized by Fillinger et al. (2008). The substitutions observed in *B. cinerea* HydR3 strains occur along the entire length of

the Erg27 protein sequence and differ from those in *B. pseudocinerea* (Fig. 14.3). They include three substitutions within or close to the NADPH binding site (at residue 23 or 26), two within the NAGI motif (at residue 196 or 199), two within the active site (at residue 336), or eight (at residue 397, 400, 408, or 412) within or close to the putative TMD (Fig. 14.3).

The F412 substitutions, the most frequent ones observed in HydR3 field strains, are predicted to potentially interfere with the helical structure of the TMD and therefore with the fenhexamid and substrate affinity to 3-KR (Billard et al. 2011, 2012a). Microsomal assays have shown that 3KR activity in HydR3$^+$ strains is only one fifth to one half that in wild-type strains, but, surprisingly, these resistant strains have a sterol profile similar to that of wild-type strains (Debieu et al. 2013). Fenhexamid-resistant mutants were generated by site-directed mutagenesis of the HydR3 *erg27* gene in a sensitive recipient strain, to overcome the impact of the different genetic backgrounds of the field strains collected. This work demonstrated that the fenhexamid resistance of HydR3 strains was entirely due to *erg27* mutation (Billard 2011; Billard et al. 2012a; Fillinger et al. 2008). This observation, together with microsomal assays of 3-KR inhibition by fenhexamid, strongly indicates that amino-acid substitutions decrease the affinity of the hydroxyanilide for the 3-KR (by factors of 20 and 100 for HydR3$^-$ and HydR3$^+$ strains, respectively), as indicated by the corresponding I_{50} values (Billard 2011; Debieu et al. 2013, and unpublished). Further studies should make it possible to identify the essential amino acids involved in the binding of the substrate (sterones) and inhibitors, such as fenhexamid or fenpyrazamine, associated with the structural changes induced by the amino-acid substitutions occurring in HydR3 strains.

14.5 Multidrug Resistance

Multidrug resistance, corresponding to the simultaneous resistance of organisms to various unrelated toxic compounds with different modes of action, is caused by an increase in the cellular efflux of these compounds. This phenomenon, which results from the overproduction of plasma membrane transporters, lowers the cytosolic concentrations of the various toxicants. In fungi, the efflux pumps involved are either ATP-binding cassette (ABC) or major facilitator superfamily (MFS) transporters (Kretschmer 2012). Multidrug-resistant (MDR) field strains displaying resistance to fenhexamid have been detected in *B. cinerea* and *O. yallundae* and were initially selected by other fungicides. In both fungi, this phenomenon is expressed principally at the conidial stage (Table 14.1) and also concerns DMIs, tolnaftate, anilinopyrimidines (e.g., cyprodinil, pyrimethanil), and novel succinate dehydrogenase inhibitors (e.g., boscalid, penthiopyrad) (Leroux et al. 1999, 2002a, 2013; Leroux and Walker 2013). In *B. cinerea*, the transporter involved is of the MFS type, and its overproduction results from a combination of the insertion of a retroelement-derived sequence and a deletion in the promoter of *bcmfsM2*. A survey conducted in French and German vineyards highlighted the existence of two

different rearrangements that have occurred separately, possibly once in Champagne and then spreading to *B. cinerea* populations from other regions (Kretschmer et al. 2009; Mernke et al. 2011). The *bcmfsM2* deletion in MDR strains results in a phenotype of susceptibility to all the fungicides concerned, including fenhexamid (Kretschmer et al. 2009). Moreover, verapamil, a known modulator of ABC transporters, acts in strong synergism with fungitoxicants in MDR fungi overproducing BcmfsM2, suggesting that this molecule may also inhibit MFS transporters (Leroux and Walker 2013). The transporter involved in multidrug resistance in *O. yallundae* has yet to be identified (Leroux et al. 2013).

14.6 Conclusions

The SBI fungicides blocking C-4 demethylation (i.e., fenhexamid and fenpyrazamine) are characterized by a narrow spectrum of antifungal activity. This is essentially due to major structural differences between fungal 3-ketoreductase (Erg27) enzymes. This enzyme is one of the three enzymes involved in sterol C-4 demethylation and is the target of fenhexamid and fenpyrazamine. Acquired resistance resulting from qualitative modifications of the 3-ketoreductase (HydR3 strains) was recorded in *B. cinerea* after the introduction of this class of SBIs. Resistance management and monitoring are required, to restrict the selection of such strains. For instance, in French vineyards, restrictions have been imposed such that only one treatment of gray mold with 3-ketoreductase inhibitors is permitted per season (Walker et al. 2013). The characterization of HydR3 strains, particularly for the predominant highly resistant strains with substitutions at position 412 of Erg27 (HydR3$^+$), can be achieved with biological methods at the conidial stage, in the presence of high concentrations of fenhexamid (Weber and Hahn 2011; Walker et al. 2013) or with molecular tools. Several PCR assays have been developed for the qualitative or quantitative detection of SNPs (Billard et al. 2012b; Grabke et al. 2013; Amiri and Peres 2014). A slight fitness cost associated with the HydR3$^+$ *erg27* mutation, as demonstrated in isogenic strains, may account for the moderate increase in their frequency in *B. cinerea* populations (Billard et al. 2012a). The mechanism of resistance at work in the less frequent HydR2 strains has yet to be determined, but target modification has been excluded, and it has been suggested that fungicide metabolism is involved. Strains resistant to fenhexamid were detected among the causal agents of gray mold before the release of this fungicide on the market, but they did not lead to practical resistance. In vineyards, these strains (HydR1 phenotype) correspond to the recently identified species *B. pseudocinerea,* living in sympatry with *B. cinerea* and naturally resistant to fenhexamid (Walker et al. 2011). This natural fenhexamid resistance, expressed principally at the mycelial growth stage, probably results from the detoxification of fenhexamid by a cytochrome P450 monooxygenase. Finally, in MDR strains of *B. cinerea,* fenhexamid resistance is associated with the overproduction of a membrane transporter of the MFS type (Kretschmer et al. 2009). A similar phenomenon probably occurred in *O. yallundae*,

the causal agent of cereal eyespot (Leroux et al. 2013). As resistance factors are low, no loss of performance in the field due to this mechanism has ever been reported for agricultural fungicides. However, the use of full doses of the individual products and their rotation are recommended. In France, fungicides are rotated between seasons.

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