Chapter 10
Chemical Control and Resistance Management of *Botrytis* Diseases

Sabine Fillinger and Anne-Sophie Walker

Abstract Chemical control remains the easiest way to manage *Botrytis* epidemics on many crops. Nevertheless, actual concerns about the environment, human health and control sustainability invite to a smarter use of fungicides, aiming to delay resistance evolution in pathogen populations. This chapter deals with the mode of action of botryticides (including multi-site toxicants and molecules affecting specifically respiration, cytoskeleton, osmoregulation, sterol and amino-acid biosynthesis) and associated resistance cases, mostly due to target site modifications. We also present original resistance mechanisms for fungi such as detoxification and multidrug resistance. Finally, this chapter introduces strategies available to decrease selection pressure exerted by fungicides on *Botrytis* spp. populations with the long-term aim to improve resistance management in the field.

Keywords Fungicides • Mode of action • Resistance mechanism • Efficacy • Strategy

10.1 Introduction

Plant diseases due to infections by *Botrytis cinerea* and other *Botrytis* species, if uncontrolled, may account for important crop losses, pre- and postharvest, with potentially high economic impact as described in the previous chapters. Integrated pest-management, including resistant cultivars, prophylactic means or application of biocontrol agents, is necessary but not always sufficient or available to prevent these diseases (see Chaps. 8, 9, and 11). Chemical control based on the application of mostly synthetic fungicides, therefore constitutes the principal means of efficient and reliable crop protection against grey mould. Control of diseases due to *Botrytis* and related species (*e.g.*, *Sclerotinia*, *Monilinia*) represents roughly 8 % of the global fungicide market (Phillips and McDougall 2012). Fungicide investment may differ among crops according to their economic value, their sensitivity to *Botrytis*

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infection and their storage time. Among them, grapes constitute high value crops for grey mould control.

During the last decades, restriction in fungicide application became necessary to reduce the impact on the environment (Fenner et al. 2013) and to limit fungicide residues (Verger and Boobis 2013) on harvest, requiring optimized protection strategies. At the same time, acquired resistance to most botryticides arose in many agronomical situations, sometimes impeding field efficacy and leading to additional sprays (Brent and Hollomon 2007). Reaching a compromise between fungicide durability, human health and environment protection and valuable crop production may imply to optimize spray timing and molecule choice and to promote agro-ecological practices combining prophylaxis measures, natural regulations (*e.g.*, by the means of biocontrol agents) and conventional fungicide treatments. In this chapter, we will describe the main chemicals used against grey mould with a focus on the latest modes of action introduced; resistance phenomena will be described, as well as their occurrence in the field, with a special highlight on new resistance mechanisms discovered since the last edition of the "Botrytis" book (Leroux 2004). Finally, we will propose rules for decision-makers, to help them adapting fungicide strategies according to the risk-situation. Most information available on these important topics concern *B. cinerea*, but data about other *Botrytis* spp. will be mentioned, when available.

10.2 Botryticides: Mode of Action and Resistance

In this chapter we consider only fungicides that inhibit or reduce disease development through direct activity on the pathogenic fungus. Their *in vitro* activities are either fungicidal or fungistatic (blocking the fungal development without killing the fungus itself). Mostly preventive, only few fungicides have curative activities once the disease is installed (for review see McGrath 2004). As will be presented in the following sections, fungicides target either specifically essential cellular functions (single-site activity), or display multi-site activity, interfering with more than one cellular function. Most modern fungicides, active at low dosage, are highly specific through their single site activity. However, concomitant with this strong activity, the risk of resistance selection after target site modifications is also high, for many modes of action. Historically, at least five groups of unisite botryticides were introduced into the fungicide market and target distinct cellular functions: (i) the cytoskeleton (microtubules); (ii) mitochondrial respiration and ATP-synthesis; (iii) ergosterol biosynthesis; (iv) biosynthesis of proteins or amino acids; (v) signal transduction. No elicitor activity on the plant's defense has been reported so far for fungicides registered against grey mould. Nevertheless, biocontrol agents or natural antifungal molecules, such as polyoxins or potassium bicarbonate, are of particular interest, especially for organic farming (Chap. 9). Polyoxins are fermentation products of *Streptomyces cacaoi* var. *asoensis* that interfere with the fungal cell wall biosynthesis (competitive inhibitor of chitin synthase). This kind of fungicide has

been used on sweet basil in Israel since the early 1990s without the selection of high resistant strains (Mamiev et al. 2013).

The major active ingredients (a.i.) and the corresponding formulated trademarks registered for grey mould control are summarized in Table 10.1. Their modes of action according to the FRAC classification (Fungicide Resistance Action Committee; www.frac.info) are described in the following sections. As we will focus our detailed descriptions on the molecules introduced since 2004, we invite the reader to refer to the corresponding chapter from 2004 (Leroux 2004) for details on older fungicide categories. Resistance to fungicides may be preexisting in a fungal population at the species level (natural resistance) or it may arise in populations after fungicide selection (acquired resistance). The susceptibility of fungal isolates to fungicides is measured by growth assays on ranges of fungicide concentrations, in order to determine the concentration inhibiting fungal growth by 50 % – also called EC_{50} – or eventually the minimal inhibitory concentration (MIC). Comparing the EC_{50} values of a given strain to those of sensitive reference strains – generally those isolated before the introduction of the fungicide – allows determining its resistance factor or level (RF or RL). We propose here to consider low resistance (LR) levels for EC_{50} ratios between 2 and 20, moderate resistance (MR) for RFs between 20 and 100; high resistance (HR) would be considered for EC50 ratios >100.

When a fungal population is treated with a given fungicide, the proportion of resistant isolates – the only individuals adapted to survive – increases. The speed of this increase may be considered as a balance between the intensity of the positive selective pressure (frequency of the applications, nature of modes of action) and of the negative selection pressure, *i.e.* the resistance cost observed in the resistant isolates relative to that of the sensitive ones. The efficacy of a given fungicide may be threatened if the frequency of highly resistant isolates in the fungal population is above a critical level, specific to each situation but often estimated to 20 % (Hollomon and Brent 2009). Anti-resistance strategies aiming to reduce the incidence of resistance in fungal populations need to combine the biological risk (inherent to the fungus' life traits), the fungicide risk (inherent to the fungicide's mode of action) and the agronomic risk (reflecting cultural practices and the intensity of fungicide use) (Kuck and Russell 2006).

10.2.1 Multisite Botryticides

Multisite toxicants figure among the eldest fungicides used in agriculture with the inorganic sulfur and copper salts described already in the nineteenth century (reviewed in Russell 2005). Against *Botrytis* diseases, molecules belonging to the chemical families of chloronitriles, phtalimides, sulfamides and dithiocarbamates (*e.g.*, folpet, thiram, tolylfluanid and chlorothalonil) are still registered in many countries, as well as in mixture with unisite fungicides, targeting numerous fungal pests including oomycetes. Most of the compounds cited in Tables 10.1 and 10.2, have highly reactive electron-rich groups with a potentially strong action on thiol

Table 10.1 Major fungicides against grey mould: mode of action, intrinsic toxicity and application rates

Fungicide			EC_{50} value (mg/l)			
Mode of action	Common name	Selected trade names	Conidia germination	Germ tube elongation	Mycelium growth	Application rate (g/ha)
Multisite	Dichlofluanid	Elvaron, Euparen	0.20	0.05	3.00	2,000
	Folpet	Folpan	3.00	0.40	≥10.00	1,500
	Thiram	Pomarsol, Thiram	≈1.00	0.10	≥10.00	2,000–3,000
	Chlorothalonil	Fongistop	0.2	0.07	3.5	1,000
Respiration	Fluazinam	Frownicide, Shirlan, Sekoya	0.10	0.04	0.08	750
	Azoxystrobin[a]	Amistar, Heritage	0.20	0.10	0.20	200
	Pyraclostrobin[a]	Pristine[b]	0.015	0.006	0.02	100–200
	Boscalid	Endura, Cantus, Pristine[b]	0.50	0.05	0.40	500–600
	Fluopyram	Luna privilege	0.50	0.05	0.60	300
Cystosqueleton	Carbendazim	Bavistin, Derosal	>10.00	0.04	0.03	500
	Thiophanate-methyl	Topsin	>10.00	0.35	0.15	1,120
	Diethofencarb[e]	Sumico[c], Jonk[c]	>10.00	0.08	0.04	500
Osmoregulation	Iprodione	Kidan, Rovral	2.00	0.80	0.15	750
	Procymidone	Sumisclex, Sumilex	2.50	0.80	0.15	750
	Fludioxonil	Geoxe, Switch[d]	0.06	0.015	0.004	500

Amino-acid synthesis	Cyprodinil	Switch[d]	≈0.10	0.008	0.01	375
	Mepanipyrim	Japica, Cockpit	≈0.25	0.03	0.04	400–600
	Pyrimethanil	Scala	≈0.30	0.05	0.08	400–800
Sterol synthesis	Prochloraz	Octave, Sportak	>10.00	0.03	0.10	250
	Tebuconazole	Horizon	>10.00	0.20	0.30	250
	Fenhexamid	Teldor, Lazulie	>10.00	0.05	0.01	750
	Fenpyrazamine	Prolectus	>10.00	0.15	0.03	600

Adapted from Leroux (2004)

[a]EC_{50}s estimated with addition of SHAM to inhibit the alternative oxidase

[b]Only in the mixture of boscalid and pyraclostrobin

[c]Mixture of carbendazim and diethofencarb

[d]Mixture of fludioxonil and cyprodinil

[e]EC_{50} values of $benA^{E198A}$ genotype (Table 10.2)

Table 10.2 Mode of action of botryticides and resistance phenomena recorded in *Botrytis* spp. field strains

Mode of action	Chemical family	Fungicides	Specific resistance			Selected references[f]
			Phenotype[a]	Target gene	Resistance allele	
Multisite	Chloronitriles	Chlorothalonil[c]	–	Unknown	–	1–3
	Phthalimides	Captan[c], folpet[c]	–	Unknown	–	
	Sulfamides	Dichlofluanid[c], tolylfluanid[c]	LR	Unknown	–	4–6
	Dithiocarbamates	Thiram[c], mancozeb[c], maneb[c]	LR	Unknown	–	2, 7, 8
Cyto-squeleton[e]	Thiophanates-benzimidazoles (Ben)	Thiophanate-methyl, carbendazim[c]	Ben^{HR} NPC^{S}	*benA* (*tubA*/*btuB*/*mbc1*)	E198A/V	9–18
	N-phenyl-carbamates (NPC)	Diethofencarb[c]	$Ben^{MR/HR}$ NPC^{R}	*benA* (*tubA*/*btuB*/*mbc1*)	F200Y	
					E198K	
Osmotic signal transduction	Dicarboximides (Imi)	Iprodione, procymidone[c], vinclozolin[c]	Imi^{LR} Phe^{S}	*bos1* (*daf1*)	I365N/R/S, V368F[b], Q369P[b], N373S[b], T447S[b], $S1040^{STOP}$	19–26
	Phenylpyrroles (Phe)	Fludioxonil	–	Unknown	–	
Methionine biosynthesis	Anilinopyrimidines	Cyprodinil, pyrimethanil, mepanipyrim	MR-HR	Unknown	–	27–29
Sterol C4-demethylation	Hydroxyanilides (Hyd)	Fenhexamid, fenpyrazamine[d]	HR ($HydR3^{+}$)	*erg27*	F412S/I/V/C,	30–36
			MR ($HydR3^{-}$)	*erg27*	F26S T63I, V192L, L195F, N196T, I232M, P250S, V309M, A314V, S336C, N369D, L400F/S, F427V A461S	
			LR-MR (HydR2)	Unknown, close to *bik*	–	37

Respiration: uncoupler	Pyridinamines	Fluazinam	LR/MR	Unknown	–	38, 39
Respiration: complex II	Pyridine carboxamides	Boscalid (Bos)	Bos^{HR} $Fpm^{S/HS}$	*sdhB*	H272Y/R	40–46
			$Bos^{MR/HR}$ $Fpm^{MR/HR}$	*sdhB*	P225T/F/L, N230I, H272L/V	
	Pyridinyl-ethyl-benzamides	Fluopyram[d] (Fpm)	Bos^{MR} $Fpm^{LR/MR}$	*sdhD*	H132R	
			$Bos^{LR/MR}$ $Fpm^{LR/MR}$	Unknown	–	
Respiration: complex III	Strobilurins	Azoxystrobin, pyraclostrobin	HR	*cytb*	G143A	43, 47–52

[a]The indicated phenotypes are *S* sensitive, *LR* low resistance, *MR* moderate resistance, *HR* high resistance according to the definition cited in Sect. 10.2
[b]Mutations found in combination with each other or with I365S
[c]Old fungicides which may not be registered any more in some countries or to protect some crops
[d]Recent fungicides which may not be already registered in all countries and on all crops
[e]*B. cinerea* wild-type phenotype is Ben^{S} NPC^{Rf}1= Corbett et al. 1984; 2= Tremblay et al. 2003; 3= Zhang et al. 2009; 4= Malathrakis 1989; 5= Pollastro et al. 1996; 6= Rewal et al. 1991; 7= Delen et al. 1984; 8= Roberts et al. 1999; 9= Bollen and Scholten 1971; 10= Leroux and Clerjeau 1985; 11= Leroux et al. 2002b; 12= Nakazawa and Yamada 1997; 13= Park et al. 1997; 14= Davidse and Ishii 1995; 15= Yarden and Katan 1993; 16= Zhao et al. 2010; 17= Banno et al. 2008; 18= Kim et al. 2009; 19= Faretra and Pollastro 1991; 20= Fujimura et al. 2000; 21= Cui et al. 2004; 22= Ma et al. 2007; 23= Carisse and Tremblay 2007; 24= Vignutelli et al. 2002; 25= Rosslenbroich and Stuebler 2000; 26= Oshima et al. 2006; 27= Fritz et al. 2003; 28= Forster and Staub 1996; 29= Bardas et al. 2008; 30= Albertini and Leroux 2004; 31= Billard et al. 2012; 32= Fillinger et al. 2008; 33= Leroux et al. 2002a; 34= Grabke et al. 2013; 35= Debieu et al. 2013; 36= Debieu and Leroux in press; 37= Schumacher et al. 2013; 38= Guo et al. 1991; 39= Tamura 2000; 40= Veloukas and Karaoglanidis 2012; 41= Kim and Xiao 2010; 42= Yin et al. 2011; 43= Leroux et al. 2010; 44= Laleve et al. 2014b; 45= Veloukas et al. 2014; 46= De Miccolis Angelini et al. 2014; 47= Ishii et al. 2009; 48= Ishii et al. 2007; 49= Banno et al. 2009; 50= Yin et al. 2010; 51= Yin et al. 2012; 52= Vallieres et al. 2011

(SH-) groups of fungal enzymes, inhibiting their reducing activity and/or the formation of disulfur-bonds (Corbett et al. 1984; Bernard and Gordon 2000). Resistance to multisite fungicides has been observed only in a few cases in *Botrytis* spp. and seems to involve detoxification (reviewed in Leroux 2004). Although less exposed to resistance development than unisite fungicides, some multisite toxicants might be withdrawn from certain countries or markets for toxicological reasons after their evaluation for re-registration, due to the high application rates necessary for these contact fungicides with solely preventive activity.

10.2.2 Unisite Fungicides

10.2.2.1 Cytoskeleton Inhibitors

The first systemic fungicides synthesized by the chemical companies were those affecting the cytoskeleton (*i.e.*, benzimidazoles, thiophanates, and N-phenylcarbamates) through microtubular binding, with severe effects on cell division, mitosis and protein secretion (Gessler et al. 1981; Temperli et al. 1991; Jochova et al. 1993; Pedregosa et al. 1995; Davidse and Ishii 1995). The N-phenylcarbamate diethofencarb mainly used against grey mould and the benzamide zoxamide, an anti-oomycete, display a similar mode of action. They were the first fungicides with curative activity against many fungal diseases, but due to massive application, most fungi including *Botrytis* spp. became resistant to these unisite fungicides, especially towards benzimidazoles and thiophanates (Bollen and Scholten 1971; reviewed in Leroux 2004) (Table 10.2). Here and at later instances of this chapter, we will not use the nomenclature of the observed phenotypes, as these may be different among authors.

Two major phenotypes of resistance to cytoskeleton inhibitors have been described for *B. cinerea*. In the first one high resistance to benzimidazoles is associated with increased sensitivity to N-phenyl-carbamates and to zoxamide (Ben^{HR}, NPC^{S}). The second phenotype displays positive cross-resistance towards the three categories of cytoskeleton inhibitors ($Ben^{HR/MR}$, NPC^{R}). In both cases, point mutations in the β-tubulin encoding gene *benA* (synonymous of *tubA*/*btuB*/*mcb*) are responsible for theses phenotypes. The amino-acid changes E198A/V were observed in the Ben^{HR}, NPC^{S} strains and E198K/L or F200Y replacement in $Ben^{HR/MR}$, NPC^{R} strains (Yarden and Katan 1993; Park et al. 1997; Banno et al. 2009; Zhang et al. 2010; Ziogas et al. 2009; Kim et al. 2009). Probably with a low resistance cost, the E198A mutants are widely distributed among *B. cinerea* populations even in the absence of selection pressure. This contrasts with those harbouring the F200Y mutation whose frequency rapidly decreases when the application of the mixture between carbendazim and diethofencarb is stopped (Walker et al. 2013). At last, resistance to benzimidazoles was detected in *B. alii*, *B. elliptica*, and *B. tulipae* (Hsiang and Chastagner 1991, 1992). Due to high efficacy losses linked with resistance selection, in many situations, and the development of other botryticides with higher intrinsic activity, anti-microtubules have now little use.

10.2.2.2 Fungicides Affecting Signal-Transduction (Osmoregulation)

Two chemical categories, applied against *Botrytis* infections, interfere with the fungal signal transduction: the dicarboximides and the phenylpyrroles, which are structural analogs of the natural antifungal compound pyrrolnitrin (Chap. 9). The exact targets of dicarboximides (*e.g.* iprodione, vinclozolin, procymidone) and the phenylpyrrole fludioxonil are still unknown. Nevertheless, these botryticides induce physiological changes, characteristic of an over-stimulation of the stress response signal-transduction (for details, see Chaps. 13 and 14), namely glycerol-accumulation, lipid peroxidation, plasma membrane leakage (reviewed in Tanaka and Izumitsu 2010; Hayes et al. 2014). They inhibit conidial germination and mycelial growth of a variety of plant pathogenic fungi (Leroux 1996). Due to extensive use, dicarboximides rapidly lost their efficiency against grey mould after the selection and generalization of specific resistance among *B. cinerea* populations (refer to Leroux 2004). Only very restricted applications of these botryticides are allowed on some crops to limit the selection of dicarboximde resistant strains, which seem to exhibit high resistance cost in the field as well (Walker et al. 2013). Resistance to dicarboximides was also easily found in *B. squamosa* on onion and on *B. elliptica* on flower bulbs in Canada but the resistance mechanism was not explored (Hsiang and Chastagner 1992; Carisse and Tremblay 2007). On the opposite, the phenylpyrrole fludioxonil does not suffer real resistance problems since only rare cases of specific resistance have been reported in *Botrytis* isolates (Vignutelli et al. 2002; Zhao et al. 2010; Ma et al. 2007). This is probably not only due to resistance management, but rather to the strongly affected fitness of fludioxonil resistant mutants. The analysis of laboratory induced fludioxonil mutants revealed reduced conidiation rate and pathogenicity, increased sensitivity to osmotic and other stresses associated with high resistance levels to fludioxonil and cross-resistance to dicarboximides in a phenotype (Viaud et al. 2006; Ma et al. 2007; Fillinger et al. 2012).

The majority of *B. cinerea* field or laboratory mutants resistant to dicarboximides and/or fludioxonil harbor mutations in the histidine-kinase gene *bos1* (syn.: *daf1*; Table 10.2). The Bos1 protein probably senses the fungicides and transmits this signal to the downstream MAP-kinase BcSak1 (and potentially other pathways), thereby stimulating the cellular response leading to cell wall breakdown, cell swelling and burst (Liu et al. 2008; reviewed in Tanaka and Izumitsu 2010).

The modifications observed in the Bos1 protein either completely abolish its function (loss of function) leading to cross-resistance between fludioxonil and dicarboximides, mostly observed in laboratory mutants, or they interfere with the N-terminal, helical HAMP-domains of the protein involved in signal transduction. Indeed the replacements of hydrophobic residues in these domains (*e.g.*, I365S) are thought to abolish their helical structure and consequently signal transduction (Fillinger et al. 2012). If the histidine-kinase Bos1 constitutes the target of either the dicarboximides or the phenylpyrroles still remains unknown. Pillonel and Meyer (1997) showed differences in protein kinase inhibition profiles between phenylpyrrole and dicarboximide fungicides. It may be, as suggested by Hayes et al. (2014) that both fungicides induce cell death through over-stimulation of the BcSak1 MAP-kinase.

10.2.2.3 Inhibitors of Amino-Acid Biosynthesis

The anilinopyrimidines mepanipyrim, pyrimethanil and cyprodinil are registered against grey mould on various crops, *solo* or in mixture with fludioxonil. They are suspected to inhibit amino-acid biosynthesis, especially that of methionine (Fritz et al. 1997). However, enzyme assays could not prove any effect of pyrimethanil on cystathione-β-lyase activity (Fritz et al. 2003) and no specific mutations were recorded, in resistant strains, either in the sequence of the corresponding gene *BcmetC*, nor in those encoding cystathionine γ-synthase, cystathionine γ-lyase, or cystathionine β-synthase, also involved in methionine synthesis (Sierotzki et al. 2002; De Miccolis Angelini et al. 2012). Therefore, the direct target of anilinopyrimidines remains unknown.

Isolates displaying moderate or high resistance to anilinopyrimidines were found a few years after the introduction of these molecules (Leroux et al. 2002b). This specific resistance is conferred by a single gene and may be suspected as target site mutation (Chapeland et al. 1999). Additional genetic analyses conducted by De Miccolis Angelini et al. (2012) indicated strong instability of anilinopyrimidine resistance during vegetative growth without selective pressure, suggesting most resistant isolates to be heterokaryons. This was confirmed by the lethality of homokaryotic anilinopyrimidine resistant ascospores. Anilinopyrimidine resistance is detected in most grey mould populations. Resistance management, restricting their application allows maintaining acceptable resistance frequencies, and efficacy, while enabling negative selection pressure to operate (Walker et al. 2013).

10.2.2.4 Ergosterol Biosynthesis Inhibitors (SBIs)

Since ergosterol is specific to the fungal kingdom and the major sterol present in the membranes of most fungi, its biosynthesis constitutes an important target for general fungicides. Despite the number of active ingredients acting as SBIs, grey mould control relies on two to four molecules, the C4-demethylation inhibitors fenhexamid (late 1990s) and fenpyrazamine (2012) and, to a lesser extent, the 14α-demethylation inhibitors (DMI), tebuconazole and prochloraz. The C4-demethylation inhibitors have a spectrum of activity limited to *Botrytis* and closely related species (Rosslenbroich 1999; Debieu et al. 2013), but *Botrytis pseudocinerea* is naturally resistant to fenhexamid (Leroux et al. 2002a; Walker et al. 2011).

The hydroxyanilide fenhexamid and the amino-pyrazolinone fenpyrazamine inhibit the 3-ketoreductase of the C4-demethylation complex, stopping ergosterol synthesis and leading to the accumulation of toxic intermediates (Debieu et al. 2001; Tanaka, Botrytis Symposium 2013, oral comm.). The selectivity of these molecules can be explained by differential affinities of fenhexamid towards the 3-ketoreductase target enzyme of different fungal species (Debieu et al. 2013).

Genetic studies have shown that acquired resistance to fenhexamid (and also to fenpyrazamine prior to its introduction) in *B. cinerea* is due to target modifications

in most strains (Fillinger et al. 2008; Billard et al. 2012). The principal highly resistant strains display a replacement of the phenylalanine at position 412 in the Erg27 protein, whereas 20 single modifications have been identified in moderately resistant strains (Albertini and Leroux 2004; Esterio et al. 2011; Fillinger et al. 2008; Grabke et al. 2013; Saito et al. 2014). These modifications decrease the affinity of fenhexamid for the 3-ketoreductase isoenzymes (Debieu et al. 2013), allowing the enzyme to be active even at high fenhexamid concentrations. Although specific resistance arose in populations a few years after fenhexamid registration, no or low efficacy losses are recorded for this molecule, possibly because the restrictions of use (*e.g.* one yearly on grapevine) keep resistant strains at an acceptable frequency and also because a low to moderate cost entails the fitness of resistant isolates (Billard et al. 2012).

10.2.2.5 Fungicides Affecting Fungal Respiration

Other essential cellular functions targeted by synthetic fungicides are fungal respiration and energy production. Eukaryotic cells use the mitochondrial electron transport chain (ETC) to oxidize the coenzyme NADH through electron exchange, but most importantly, the electrochemical proton gradient produced across the inner mitochondrial membrane allows the production of ATP, the cellular energy source necessary for any metabolic activity. As outlined in Fig. 10.1, the ETC is composed of four enzymatic complexes, involved in electron exchange through redox reactions, and the final enzyme, ATP synthase. Complex II, namely succinate dehydrogenase (SDH), also has an enzymatic function in the tricarboxylic acid (TCA) cycle. Five functional categories of respiration inhibitor fungicides have been developed, and three of them are registered as botryticides (Fig. 10.1): those inhibiting complex II (SDHIs), complex III (QoIs) and uncouplers of oxidative phosphorylation.

Uncouplers

Uncouplers reduce the proton-gradient across the mitochondrial membrane and therefore decrease or even inhibit ATP synthesis (Russell 2005). External uncouplers are generally hydrophobic compounds with a delocalized negative charge, which penetrate the mitochondrial membrane. The sole fungicide, classified as uncoupler acting on oxidative phosphorylation, is the dinitro-aniline – or pyridine – amine – fluazinam with a broad spectrum of preventive activity, used in particular against oomycetes and grey mould (reviewed in Terada 1981; Kadenbach 2003). It acts as uncoupler involving protonation/deprotonation reactions due to a protonophoric cycle (Brandt et al. 1992). Several authors suggested additional activities for fluazinam on mitochondrial respiration, *e.g.* inhibition of thiol groups (Brandt et al. 1992), release of cytochrome c into the cytosol and inhibition of complex I of the

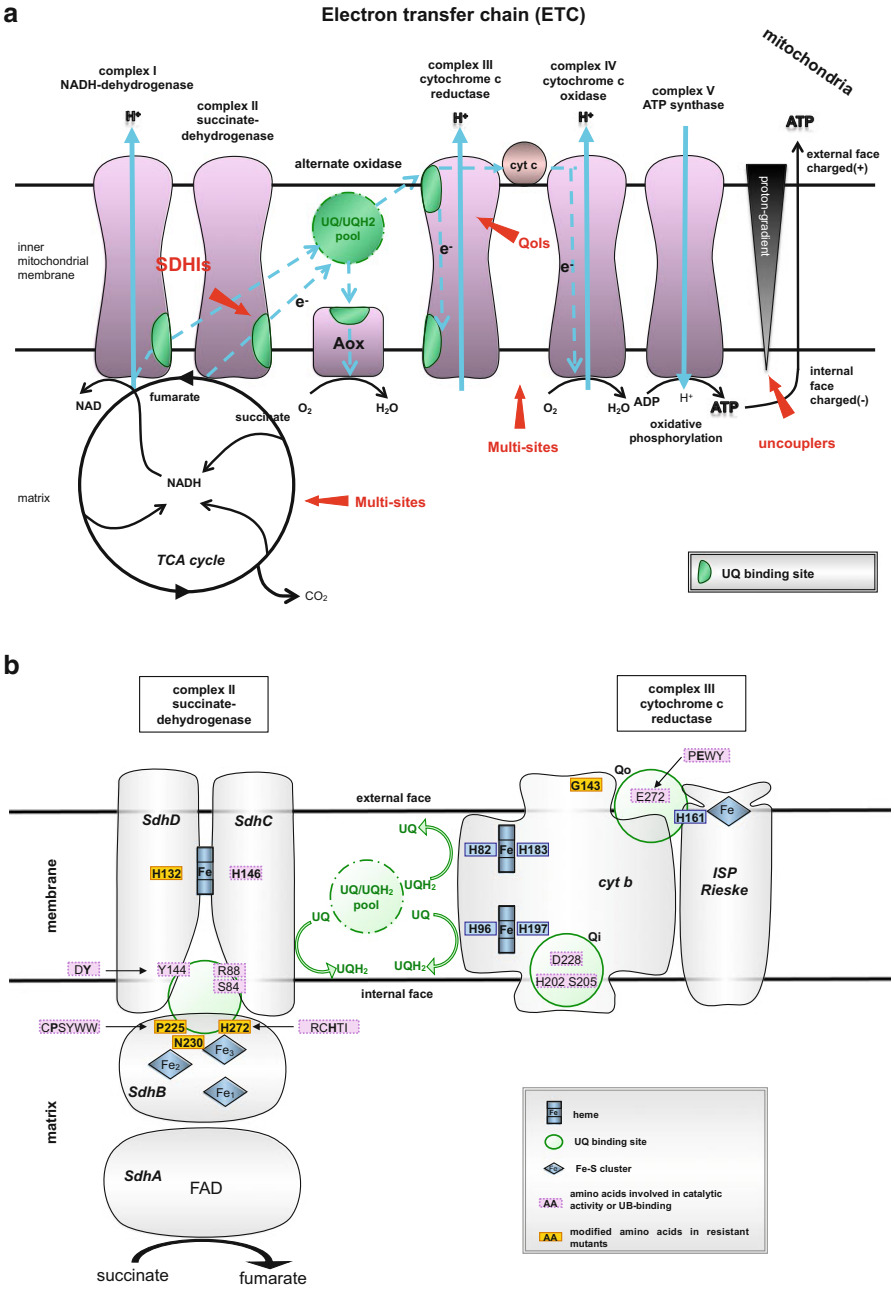
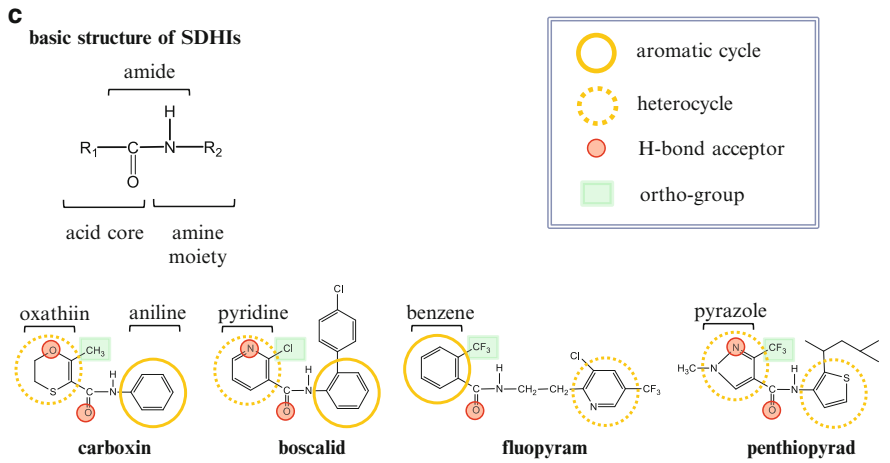


Fig. 10.1 Targets of respiration inhibitors and modified residues involved in resistance with focus on complex II inhibitors (SDHIs). (**a**) Enzyme complexes and target sites of respiration inhibitors on the electron transfer chain (Adapted from Leroux and Walker 2010); (**b**) Detailed view of complexes II and III of the ETC, with the conserved residues in the catalytic or ubiquinone (*UQ*) binding site (Adapted from Leroux and Walker 2010); (**c**) Chemical structure of some SDHIs (Adapted from Glättli et al. 2011); (**d**) Resistance profile of *B. cinerea* mutants to different SDHIs – field isolates (field) compared to *sdhB* site-directed mutants (lab). When particularly high SDHI concentrations are necessary to determine the EC_{50} value, only the minimum resistance factors are indicated over the columns (>RF) (Adapted from Lalève et al. 2014b)



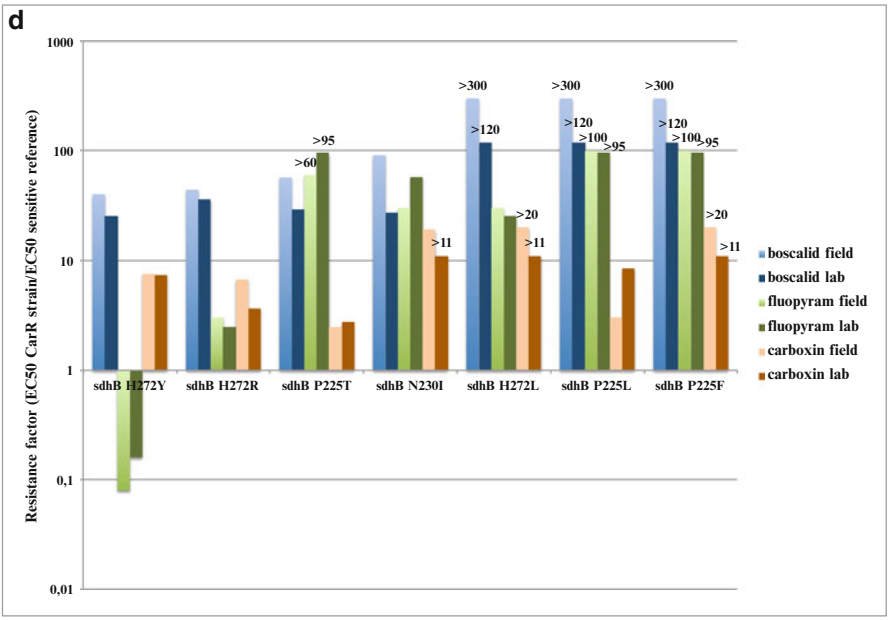


Fig. 10.1 (continued)

ETC (Akagi et al. 1996). These multiple activities on fungal respiration may explain the broad spectrum of fungitoxicity, but also why only few cases of fluazinam resistance have been reported so far for *B. cinerea* (Table 10.2). If resistance to fluazinam involves detoxication eventually through the action of GSTs (gluthathion-S-transferases), as suggested by Leroux (2004), remains to be investigated.

Inhibitors of Complex II: Succinate Dehydrogenase Inhibitors (SDHIs)

SDH couples the oxidation of succinate to fumarate in the mitochondrial matrix with ubiquinone reduction in the inner mitochondrial membrane. It is a complex of four proteins (*i.e.*, SdhA; SdhB; SdhC; SdhD) encoded by nuclear genes: the soluble entity, responsible for the succinate dehydrogenase activity of the enzyme, consists of subunits A and B; the SdhC and SdhD subunits form the integral membrane component, anchoring the enzyme complex to the inner mitochondrial membrane. The ubiquinone-binding site (Q-site) involves amino acids from SdhB, SdhC and SdhD (Cecchini et al. 2003; Hagerhall 1997). Fungicides of the carboxamide family inhibit ubiquinone reduction by binding to the Q-site of SDH. The intact carboxamide structure (R_1-CO-NH-R_2) seems to be required for full fungicidal activity. SDHIs build hydrogen-bonds (H-bonds) with the conserved residues of the Q-site through the heteroatoms highlighted in Fig. 10.1c and hydrophobic or π interactions through the aromatic cycle of the amine moiety (Glättli et al. 2011).

Six classes of SDHIs can be defined on the basis of the chemical structure of the acidic moiety of the molecule (reviewed by Leroux et al. 2010; Sierotzki and Scalliet 2013) (R_1): benzamides (*e.g.* fluopyram, flutolanil), furan carboxamides (*e.g.* fenfuram), oxathiin carboxamides (*e.g.* carboxin, oxycarboxin), pyrazole carboxamides (*e.g.* bixafen, isopyrazam, penthiopyrad), pyridine carboxamides (*e.g.* boscalid) and thiazole carboxamides (*e.g.* thifluzamide). The benzamides can also be subdivided into two groups on the basis of differences in the amine moiety (R_2): phenyl benzamides (*e.g.*, flutolanil) and pyridinyl ethylbenzamides (*e.g.* fluopyram). Actually, boscalid (2002), penthiopyrad (2009), isopyrazam (2010) and fluopyram (2012) figure among the latest registered fungicides against *Botrytis* spp., but similar molecules from other companies may be introduced (*e.g.*, benzovindiflupyr, isofetamid).

Once the baseline sensitivity to boscalid was established by different methods on *B. cinerea* isolates from different hosts and regions (Stammler and Speakman 2006; Zhang et al. 2007; Myresiotis et al. 2008), the first isolates resistant to boscalid were reported in 2007 (Stammler et al. 2007) and since then successively on many crops in several countries (Kim and Xiao 2010; Leroux et al. 2010; Yin et al. 2011; Veloukas et al. 2011; Fernandez-Ortuno et al. 2012; De Miccolis Angelini et al. 2014; Amiri et al. 2014 and others). The carbon source seems to be a critical issue in bioassays, as glucose may compensate SDHI toxicity and should be replaced by acetate, succinate or glycerol. As in other fungi, mutations were found in the genes encoding the subunits B and D of succinate-dehydrogenase, *sdhB* and *sdhD*, especially for the residues $SdhB^{P225}$ and $SdhB^{H272}$ of the ubiquinone binding site, or $SdhD^{H132}$ involved in heme-binding (Fig. 10.1b, d), but also the N230I modification in SdhB. Although modifications of SdhC have also been found in *B. cinerea* strawberry isolates, strict correlation with resistance to boscalid and fluopyram could not be found (Mosbach et al. 2014).

The highest levels of resistance have been recorded for the $SdhB^{P225F/L}$ and $SdhB^{H272L/V}$ substitutions. $SdhB^{H272R}$ and $SdhB^{H272Y}$ are the most frequently detected substitutions in boscalid-resistant strains. Genetic analyses and site directed

mutagenesis showed that these modifications of SdhB confer boscalid resistance (De Miccolis Angelini et al. 2010; Laleve et al. 2014b). In fact, they are responsible for different levels of resistance to this pyridine carboxamide, but also for different spectra of cross-resistance to fluopyram, to the oxathiin carboxamide carboxin and to other SDHIs (Leroux et al. 2010; Veloukas et al. 2014) (Fig. 10.1d). Lalève and colleagues (2014b) showed for the *sdhB* mutations a strong correlation between the affinity of SDHIs for the SDH isoforms, SDH inhibition and *in vivo* growth inhibition confirming the key roles of H272, P225 and N230 in carboxamide binding (reviewed in Sierotzki and Scalliet 2013; Laleve et al. 2014b). The $sdhB^{H272Y}$ mutation, leading to fluopyram hypersensitivity, had no effect on SDH activity or respiration. This category of SDHI-resistant mutants, which is currently the most frequently isolated in many agronomic situations, may therefore be well controlled by alternating or mixed applications of boscalid and fluopyram, at least in the coming years. Resistance to SDHIs is associated with fitness cost, either on field mutants (Veloukas et al. 2014) or on isogenic laboratory mutants (Laleve et al. 2014a). Despite discrepancies between the results, both studies revealed more or less important fitness penalty on several life traits linked to the *sdhB* mutations. Veloukas and colleagues' competition assays (2014) on apple between SDHI-resistant and sensitive strains showed clear differences according to the selective pressure. In the presence of fluopyram, for example, the $sdhB^{P225F}$ isolates dominated the population.

The concurrent use of boscalid and fluopyram (and also of future SDHIs) could change the structure of resistant populations, favoring already known or new *sdh* alleles conferring strong positive cross-resistance between all molecules (Amiri et al. 2014). Continuous monitoring studies either with biological assays or combined with molecular tools (De Miccolis Angelini et al. 2014) are necessary to evaluate the impact of variations in SDHI selection pressure on resistance development.

Inhibitors of Complex III (QoIs)

The last two decades was the period of "raise and fall" of strobilurins on many crops. Synthetic molecules derived from the secondary metabolite strobilurin A, produced by basidiomycetes such as *Strobilurus tenacellus*, have been introduced on the fungicide market since 1992 (Russell 2005). They bind to the quinol oxidation (Qo) site of cytochrome b (complex III of the ETC) and thereby stop electron transfer between complex III and IV, inhibiting NADH oxidation and ATP synthesis in many fungal pathogens (reviewed in Balba 2007). Strobilurins are referred to as QoI fungicides, as they bind to the inner Qo-site (Fig. 10.1b). Two QoIs (azoxystrobin and pyraclostrobin), often associated to other modes of action, were used on several crops to control *Botrytis* disease and other fungi at the same time (Table 10.1). Indeed, QoIs have low intrinsic activity on *Botrytis* sp., due to the constitutive expression of the terminal alternative oxidase (AOX). AOX allows electrons to bypass the blockage of the cytochrome pathway caused by strobilurins (Ishii et al. 2009).

This category of fungicides bares a high risk of resistance development, as the target, cytochrome b, is encoded by a mitochondrial gene: *cyt b* mutations responsible for resistance (Table 10.2) confer resistance also under heteroplasmic conditions and are maternally transmitted, but probably also through hyphal fusion (reviewed in Gisi et al. 2002; Villani and Cox 2014; De Miccolis Angelini et al. 2012). Most phytopathogenic fungi treated with QoIs became resistant through the acquisition and dispersal of the G143A and two other minor mutations in the *cyt b* gene (Gisi et al. 2002; Russell 2005). Although in some *B. cinerea* strains the presence of an intron located precisely at codon 143 counter-selects the G143A mutation (Banno et al. 2009; Ishii et al. 2009; Leroux et al. 2010; Jiang et al. 2009; Asadollahi et al. 2013; Vallieres et al. 2011), QoI resistance is now generalized in *Botrytis* populations (due to the presence of resistance phenotype even in heteroplasmic cells), in agreement with the lack of fitness penalty associated with the *cyt* b^{G143A} allele (Veloukas et al. 2014). This resistance was also generalized on crops (*e.g.*, grapevine) that never received QoIs against *Botrytis* spp., suggesting that it can be unintentionally selected *via* sprays targeting other diseases. Considering the QoI resistance risk in *Botrytis* spp. and the limited intrinsic activities of these molecules, those should be replaced, whenever possible, by specific botryticides efficient on local populations. Finally, a novel QoI, the benzylcarbamate pyribencarb, with promising efficiency on QoI resistant strains, due to poor cross-resistance with strobilurins, is actually in the registration process (Takagaki et al. 2011). Indeed, it was suggested that pyribencarb might differ slightly in the binding sites within cytochrome b, compared to other QoIs (Kataoka et al. 2010).

10.2.3 Resistance Mechanisms Unlinked to the Target

Besides specific resistance to given fungicides due to target site modification, several other mechanisms have been extensively studied in *Botrytis* spp. in the last decade, eventually conferring cross resistance to unrelated chemical compounds, because the principal mechanism induces the reduction of the intracellular concentration of toxic compounds.

10.2.3.1 Multi-drug Resistance

Botrytis cinerea isolates displaying monogenic low-to-moderate resistance to several fungicides have been detected in French vineyards since the 1990s (Chapeland et al. 1999), probably due to the high concomitant selective pressure of various chemical families. Three patterns of cross-resistance were described (Leroux et al. 1999; Leroux and Walker 2013), respectively named MDR1, MDR2 and MDR3. All display cross resistance (low to medium RLs) to anilinopyrimidines, diethofencarb, iprodione, fludioxonil, some respiration inhibitors, but also to the

clinical sterol-biosynthesis inhibitor tolnaftate (high RLs). Additional resistances allow distinguishing between MDR1 and MDR2 strains as shown in Fig. 10.2, while MDR3 strains combine resistance spectra of both phenotypes with additive resistance factors.

Multi-drug resistance (MDR) is a well-known phenomenon in the medical sector. Generally due to increased efflux of unrelated toxic compounds, it involves the upregulation of membrane transporters, either ABC (ATP-binding cassette) transporters or those of the major-facilitator superfamily (MFS) (for reviews see Moye-Rowley 2003; Morschhäuser 2010). Fungicide efflux is also at work in *B. cinerea* MDR strains correlated to membrane transporter overexpression. While MDR1 strains show overexpression of the ABC transporter gene *atrB* linked to single modifications in the transcription factor Mrr1, the MDR2 phenotype is due to *mfsM2* overexpression, itself originating from the insertion of a retrotransposon like element. MDR3 strains derive from recombination of both *mdr* mutations, probably after sexual crosses (Kretschmer et al. 2009; Mernke et al. 2011). In Champagne vineyards, more than 60 % of collected grey mould populations display an MDR phenotype (Walker et al. 2013), approximately 20 % of each phenotype. Despite this high frequency, no loss in field efficacy is observed with current fungicides at recommended application rates, as the resistance factors of MDR strains are low to moderate (Fig. 10.2).

In German strawberry fields the situation seems more severe with large proportions of MDR strains cumulating specific resistance(s) due to target site mutations, therefore leading to high resistance levels to many fungicides. In addition, the strawberry specific *B. cinerea* group S (described in Chap. 6) contains a new MDR1 phenotype named MDR1h, with two to three times higher resistance levels than previously identified MDR1 strains to cyprodinil and fludioxonil. A 3 bp deletion in the *mrr1* gene (Δ^{L497}) leads to 150–300 fold overexpression of *atrB*, three to six times higher than in MDR1 strains (Leroch et al. 2013). The combination of *mdr* mutations with specific resistance alleles may lead to serious crop losses, if the frequency of highly multi-resistant strains reaches a certain threshold and if their fitness is not too much affected.

10.2.3.2 Detoxification

Detoxification of chemical drugs through enzymatic metabolisation involving gluthathion-S-transferases (GSTs), cytochrome P450s, hydrolases or esterases constitutes a resistance mechanism widespread in insect pests and weeds (Delye et al. 2013; Ffrench-Constant 2013). Such as MDR, detoxification can confer cross-resistance to pesticides with different modes of action. In phytopathogenic fungi, this mechanism has been rarely involved in fungicide resistance. Some *B. cinerea* strains have been shown to be sensitive to the anti-oomycete fungicide cymoxanil through metabolic activation of this profungicide (Tellier et al. 2008, 2009). This phenomenon does not interfere with grey mould control, as this compound is not used against *Botrytis* spp. Detoxification was proposed as a possible resistance

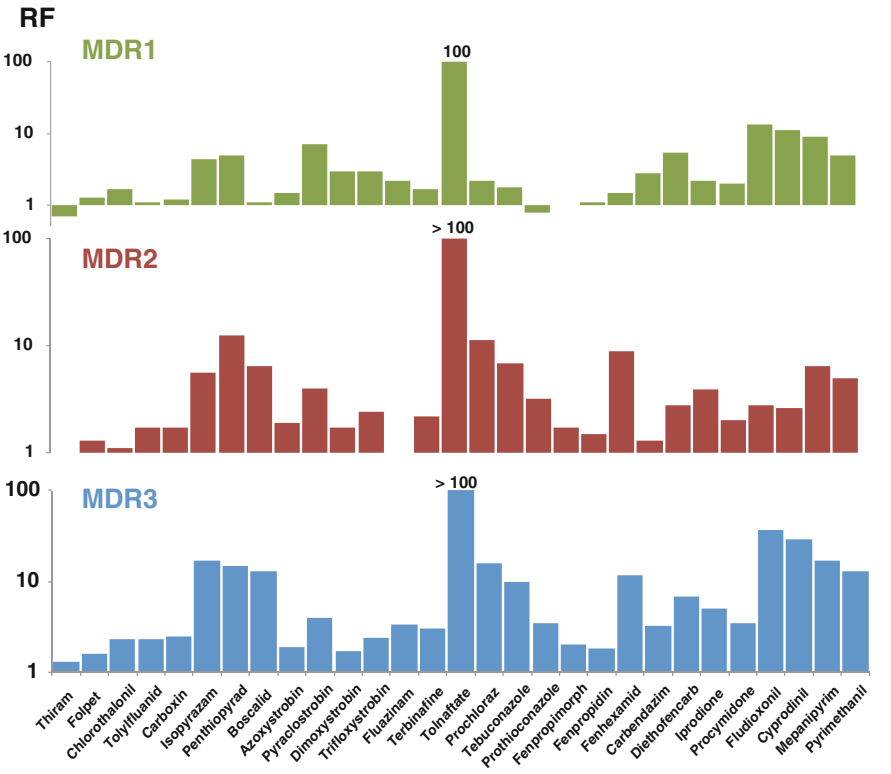


Fig. 10.2 Resistance factors of *B. cinerea* MDR strains on various fungicides (Adapted from Leroux and Walker 2013)

mechanism against multisite fungicides and fluazinam, as described in the review of Leroux (2004), but only few field isolates resistant to these compounds have been found so far.

The natural resistance of the new species *B. pseudocinerea* (Chap. 6) to the hydroxyanilide fenhexamid (formerly known as HydR1 phenotype) seems to involve detoxification. Besides the reduced affinity of fenhexamid for its target enzyme in *B. pseudocinerea* compared to the *B. cinerea* enzyme (Debieu et al. 2013), Leroux and colleagues observed synergy between fenhexamid and DMIs on *B. pseudocinerea*'s mycelial growth (Leroux et al. 2002a) and studies conducted by Bayer SAS showed metabolisation of fenhexamid by *B. pseudocinerea* strains (Suty et al. 1999). Later, Billard et al. identified a cytochrome P450 similar to the DMI target Cyp51, named Cyp684, whose inactivation nearly completely abolished fenhexamid resistance in *B. pseudocinerea* (Billard et al. 2011), indicating that Cyp684 is a major player in *B. pseudocinerea*'s natural resistance, potentially through fenhexamid metabolisation. The expression of *cyp684* displays higher induction levels

in *B. pseudocinerea* strains after fenhexamid treatment, than in *B. cinerea* strains, but the metabolisation products still remain unknown (Billard et al. unpublished).

A rarely observed fenhexamid resistance phenotype in *B. cinerea*, named HydR2 (see Table 10.2), seems to be linked to fungicide detoxification as well. Synergy between DMIs and fenhexamid suggests the involvement of a cytochrome P450 (Leroux et al. 2002a) and no changes in *erg27* sequence, nor its expression, were observed with HydR2 isolates (Billard, unpublished). As the above mentioned *cyp684* was excluded from HydR2 phenotype (Billard, unpublished), the cytochrome P450 involved remains to be identified. Genetic analyses indicated its genomic location close to the *bik* gene cluster involved in bikaverin biosynthesis (Schumacher et al. 2013; see Chap. 13).

10.3 Fitness Cost of Fungicide Resistance

Resistance to fungicides may be associated with a cost, as generally reported for fungal populations subjected to fungicide-mediated selection pressure (Milgroom et al. 1989). Characterization of the cost of resistance in resistant isolates may make it possible to predict the rate of evolution of such isolates in the population. This characterization is therefore essential for estimation of the extent to which resistant isolates constitute a risk to disease control by fungicides and for the optimization of anti-resistance strategies. As an example, detecting a fitness cost may be of great interest in strategies alternating fungicidal modes of action since it may substantially delay resistance evolution between two applications (REX Consortium 2013).

Fitness is the ability of an individual to survive in its environment and to contribute successfully to the next generation (Orr 2009). Differences in fitness between individuals may arise from differences in performance at any stage of the life cycle, and any variation of these fitness components can contribute to differences in total fitness between individuals (Antonovics and Alexander 1989). Fitness can be measured using two approaches. More generally research groups measure several parameters on fungicide resistant field isolates in comparison to sensitive strains. The traits generally measured for phytopathogenic fungi are conidiation, conidia germination, hyphal growth and virulence (Antonovics and Alexander 1989) and should be chosen all along the life cycle. These analyses globally hint to fitness penalties (or not) of the phenotypic category considered (Bardas et al. 2008; Saito et al. 2014; Veloukas et al. 2014), but they need to be performed on a statistically significant set of representative strains, because the genetic and phenotypic polymorphism of natural *Botrytis* isolates may hide or exaggerate the phenotype linked to the resistance allele.

An alternative approach was developed these recent years by the construction of isogenic mutants using site-directed mutagenesis through homologous gene replacement. Briefly, all mutant strains are identical except for the resistance allele. Comparing their biological features allows precisely attributing a fitness cost to each allele by *in vitro* and *in planta* measurements (Billard et al. 2012; Laleve et al. 2014a).

However, both types of analyses do not necessarily give the same results. As in the case of SDHI resistant mutants, the study of isogenic laboratory mutants revealed the highest fitness penalty for the *sdhB*^{H272R} allele (Laleve et al. 2014a), whereas the very similar analysis of field-strains gave the lowest fitness penalty to this allele among all *sdhB* alleles tested (Veloukas et al. 2014). The genetic context of the respective field and laboratory strains may influence the biological parameters of the resistant mutants. Moreover, fitness is only estimated via a limited number of life traits, which may not be relevant. Therefore, conclusions about fitness penalties to predict the risk of a given resistance to persist and spread should be drawn with precautions when using this approach.

Another set of methods tends to evaluate fitness as a whole, *i.e.* trying to measure the survival of resistant strains and the evolution of their frequency in populations. This can be approached *in vitro* or *in planta* with competition experiments, measuring the frequency of each genotype after each subculture cycle (see examples in Veloukas et al. 2013; Laleve et al. 2014a) but cannot fully mimic biotic interactions, as they may happen in the field. Total fitness can also be estimated mathematically, by modeling changes in allele frequencies in populations subject to natural selection (Orr 2009). This can be achieved while detecting cline patterns, *i.e.* a gradient of resistant allele frequency over a geographical transect. Parameters of cline models may be direct indicators of selection, either negative or positive, and of migration, as demonstrated in resistance to insecticides and fungicides (Lenormand et al. 1999; Walker and Fournier 2014). Non-spatial models, modelling the evolution of resistance frequency all along the fungus life cycle may also help inferring these parameters (Walker and Fournier 2014).

10.4 Resistance Management

Anti-resistance strategies are based on the skillful deployment of tools (prophylaxis, plant resistance genes and antifungal compounds) to delay resistance. Prophylaxis against *Botrytis*, even of partial efficacy, can be deployed in many crops and mainly deals with decreasing the plant vigor (*via* fertilization management, host density), creating a dry climate around the susceptible organs (*via* pruning, green harvest, climate regulation in greenhouses) and preventing wounds on susceptible organs (control of insect vectors, adaptation of mechanical tools) (Chap. 8). Additionally, crops may have at least partial resistance to *Botrytis* in some cultivars.

Dealing with antifungals, either synthetic or natural, several strategies are available. Firstly, fungicides may be limited in their use, as early as registration. This may be of great efficacy in decreasing the selective pressure for a given mode of action but not adapted to crops that need a large number of sprays. As fungicides often have lower intrinsic activities against *Botrytis* than against other fungi, the mixture strategy, may not be the appropriate or should be restricted to the most powerful inhibitors, *i.e.* which suffer dose reduction (for economic, environmental or toxicological reasons). As mixture is based on the redundant killing of fungal

species, both partners should be fully efficient against *Botrytis* local populations, not to expose one of the modes of action (*e.g.*, QoIs + SDHIs). Regular mixture applications may also select for generalist resistance mechanisms, such as MDR (*e.g.*, fludioxonil + cyprodinil).

Limiting the use of each botryticide at the multi-seasonal scale and alternating active ingredients at full dose with different modes of action seems to be a suitable approach in many situations, particularly in cases of emerging resistance (*e.g.* resistance to SDHIs). Indeed, this strategy allows the expression of resistance cost, as the same molecules may target distinct pathogen generations. This strategy has been shown to decrease the frequency (*e.g.*, dicarboximide or benzimidazole resistant strains) or to delay the emergence of resistant strains (*e.g.* to anilinopyrimidines or to fenhexamid), for example in French vineyards. The key elements of the actual management of fungicides resistance in *Botrytis* have been summarized as a decision tree based on the observation of mechanism, frequency and phenotype of field resistant mutants (Walker et al. 2013).

At last, as anti-resistance strategies delay but not fully prevent resistance, *Botrytis* control can only be efficient and durable if innovative modes of action are regularly released on the market. Keeping the diversity of modes of action, even with partial efficacy, is crucial in resistance management. During the last decades, important breakthroughs were achieved in the discovery of new resistance mechanisms, their genetic determinants (see also Chap. 3), the development of molecular tools to detect and quantify resistance phenomena in grey mould populations. Altogether, these achievements may help optimizing the chemical control of this threatening disease. In addition, resistance monitoring, with adapted technical procedures, relevant sampling sizes and observed areas, should accompany the fungicides' life, to identify and optimize the anti-resistance strategies to local situations.

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