Risk assessment studies on succinate dehydrogenase inhibitors, the new weapons in the battle to control Septoria leaf blotch in wheat

BART A. FRAAIJE[1],*, CARLOS BAYON[1], SARAH ATKINS[1], HANS J. COOLS[1], JOHN A. LUCAS[1] AND MARCO W. FRAAIJE[2]

[1]*Rothamsted Research, Plant Pathology and Microbiology Department, Harpenden, Hertfordshire AL5 2JQ, UK*
[2]*Biochemical Laboratory, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, the Netherlands*

SUMMARY

Chemical control of Septoria leaf blotch, caused by *Mycosphaerella graminicola*, is essential to ensure wheat yield and food security in most European countries. *Mycosphaerella graminicola* has developed resistance to several classes of fungicide and, with the efficacy of azoles gradually declining over time, new modes of action and/or improvements in host varietal resistance are urgently needed to ensure future sustainable disease control. Several new-generation carboxamide fungicides with broad-spectrum activity have recently been introduced into the cereal market. Carboxamides inhibit succinate dehydrogenase (Sdh) of the mitochondrial respiratory chain (complex II) but, because of their single-site specificity, these fungicides may be prone to resistance development. The objective of this study was to assess the risk of resistance development to different Sdh inhibitor (SDHI) fungicides in *M. graminicola*. UV mutagenesis was conducted to obtain a library of carboxin-resistant mutants. A range of SDHI resistance-conferring mutations was found in Sdh subunits B, C and D. Pathogenicity studies with a range of Sdh variants did not detect any fitness costs associated with these mutations. Most of the amino acid residues identified (e.g. B-S221P/T, B-H267F/L/N/Y, B-I269V and D-D129E/G/T) are directly involved in forming the cavity in which SDHI fungicides bind. Docking studies of SDHI fungicides in structural models of wild-type and mutated Sdh complexes also indicated which residues were important for the binding of different SDHI fungicides and showed a different binding for fluopyram. The predictive power of the model was also shown. Further diagnostic development, enabling the detection of resistant alleles at low frequencies, and cross-resistance studies will aid the implementation of anti-resistance strategies to prolong the cost-effectiveness and lifetime of SDHI fungicides.

Correspondence: Email: bart.fraaije@rothamsted.ac.uk

INTRODUCTION

The control of fungal foliar cereal diseases is mainly dependent on the deployment of resistant host cultivars and the programmed application of fungicides. Septoria leaf blotch (SLB), caused by the ascomycete *Mycosphaerella graminicola* (anamorph *Septoria tritici*), has been the most important foliar disease of winter wheat in the UK since the 1980s (Bearchell *et al.*, 2005; Hardwick *et al.*, 2001). Current commercial wheat cultivars are susceptible or moderately resistant to the disease in the UK, because of the relatively low priority of disease resistance in breeding programmes, in combination with the availability of effective systemic fungicides over the last three decades. However, this approach may change as a result of fungicide resistance evolution and the introduction of new European Union regulations on the use of plant protection products (Directive 2009/128/EC and Regulation 1107/2009) impacting on product (re-)registration and discovery. As a result of resistance development to benzimidazole fungicides (Griffin and Fischer, 1985) and quinone outside inhibitors (QoIs) (Fraaije *et al.*, 2005), in 1984 and 2002, respectively, the control of SLB is currently dependent on the use of systemic demethylation inhibitors (DMIs; azole fungicides) and the protective multi-site inhibitor chlorothalonil.

Fungicide programmes, typically two to four sprays of fungicide mixtures during the growing season, contribute to profitable farming by increasing yields by around 2 tonnes/ha, equivalent to 25% of the average UK yield. They have also been shown to reduce greenhouse gas emissions significantly by a more optimal use of nitrogen fertilizer in the absence of disease (Berry *et al.*, 2008). The sensitivity of *M. graminicola* populations to azole fungicides has been declining gradually over time, already affecting field performance for some products (Clark, 2006). As a result of the continued evolution of novel CYP51 (sterol 14α-demethylase) variants, further shifts in sensitivity towards different azoles are expected (Brunner *et al.*, 2008; Cools and Fraaije, 2008; Cools *et al.*, 2011; Leroux and Walker, 2011) and new modes of action are urgently needed to ensure future sustainable disease control.

Several new-generation carboxamide fungicides have been introduced recently [e.g. boscalid (2005), isopyrazam (2010)

and bixafen (2011)] or are expected to be launched shortly into the UK cereal market (e.g. penthiopyrad) (Fig. 1). Carboxamides inhibit succinate dehydrogenase (Sdh) of the mitochondrial respiratory chain (complex II). In contrast with early-generation Sdh inhibitors (SDHIs), such as carboxin introduced in 1969, the new compounds have a wide spectrum of activity and some curative properties. So far, resistance to SDHI fungicides has not been found in field isolates of cereal pathogens. However, carboxin-resistant laboratory mutants of *M. graminicola* have been reported (Skinner *et al*., 1998). These mutants carry amino acid substitutions H267L and H267Y in the Sdh iron sulphur protein subunit B (SdhB) and are cross-resistant to boscalid (B. A. Fraaije, unpublished). Similar or identical mutations at this codon and mutations at other positions within SdhB, and subunits SdhC and SdhD, have also been found to confer resistance to SDHI fungicides in laboratory mutants and field isolates of a range of fungi, including *Alternaria alternata* (Avenot *et al*., 2008, 2009), *Aspergillus oryzae* (Shima *et al*., 2009), *Botrytis cinerea* (Leroux *et al*., 2010; Stammler *et al*., 2008) and *Corynespora cassiicola* (Miyamoto *et al*., 2010). Interestingly, for some inhibitors (e.g. fluopyram), a lack of cross-resistance to other SDHIs was identified when resistant isolates were tested (see Avenot and Michailides, 2010; Ishii *et al*., 2011; White *et al*., 1978).

To prevent or delay resistance development to SDHI fungicides in cereal pathogens, the Fungicide Resistance Action Committee (FRAC) has recommended the use of SDHI fungicides preventatively in mixtures with a maximum of two sprays per season. Mixing partners should ideally have a different mode of action and, on their own, provide satisfactory disease control. For the control of SLB, azoles are the preferred mixing partner, and it is expected that most SDHI products will enter the UK wheat market as co-formulations with azoles.

The objective of this study was to assess the risk of resistance development to different SDHI fungicides in *M. graminicola*. We measured the baseline sensitivities to SDHIs and other fungicides in old and recent *M. graminicola* field isolates from different geographical origins. UV mutagenesis was conducted to obtain carboxin-resistant mutants. These mutants were further characterized for levels of resistance and cross-resistance patterns using *in vitro* fungicide sensitivity assays to a range of SDHIs. For a selection of mutants, pathogenicity tests were carried out to establish their fitness *in planta*. In addition, *in silico* studies on the binding of fungicides to the Sdh complex of *M. graminicola* were carried out. Docking studies of SDHIs in structural models of wild-type and mutated Sdh complexes established which residues were important for binding, and the predictive power of the model was also investigated. Knowledge from this study can be used to devise and test anti-resistance strategies to prolong the cost-effectiveness and lifetime of SDHIs.

RESULTS

Fungicide baseline sensitivity testing of *M. graminicola* field strains

Table 1 shows the fungicide sensitivities of 30 field strains representing past and recent field populations sampled from different geographical regions. Carboxin was the least effective compound amongst the SDHIs tested, with recorded EC_{50} values between 0.608 and 5.52 μg/mL and an average value of 1.84 μg/mL. Boscalid (EC_{50} between 0.161 and 1.14 μg/mL; average, 0.482 μg/mL) was less effective *in vitro* than isopyrazam (EC_{50} between 0.020 and 1.1 μg/mL; average, 0.262 μg/mL) and bixafen (EC_{50} between 0.019 and 0.664 μg/mL; average, 0.219 μg/mL). When sensitivity data from strains isolated before 2005 were compared with the sensitivities of those isolated after 2005 (coinciding with the introduction of boscalid in the UK), there were no differences in EC_{50} values for the four SDHIs and chlorothalonil. The sensitivity data for epoxiconazole and prothioconazole-desthio showed a clear trend of decreasing sensitivity for strains isolated more recently in northwest Europe, a region with a high Septoria disease pressure, compared with older strains. This change in sensitivity has been correlated with the evolution of more complex CYP51 variants harbouring multiple amino acid alterations (Stammler *et al*., 2008).

Clear linear cross-sensitivity patterns were observed for bixafen and isopyrazam, bixafen and boscalid, and isopyrazam and boscalid, with R^2 values of 0.65 ($P < 0.001$), 0.61 ($P < 0.001$) and 0.46 ($P < 0.001$) ($n = 30$), respectively. The R^2 values for carboxin and bixafen, carboxin and isopyrazam and carboxin and boscalid were lower, at 0.17 ($P = 0.022$), 0.17 ($P = 0.025$) and 0.03 ($P = 0.385$) ($n = 30$), respectively. As expected, of the remaining fungicide pairs tested, a clear linear cross-resistance relationship was only measured between the two DMI fungicides epoxiconazole and prothioconazole-desthio ($R^2 = 0.85$; $P < 0.001$; $n = 21$) after discarding the 'wild-type' strains (strains with no alterations in CYP51 or variants carrying L50S, S188N and N513K) (Leroux *et al*., 2007).

Sdh sequence variation among *M. graminicola* field strains

DNA sequence comparison between IPO323, IRE30 and five other strains (BC1, BC4, R35-3, R39-1 and V212-2) showed that the Sdh subunits were generally well conserved (GenBank accession numbers JF916683–916700). For SdhB, only changes resulting in the replacement of lysine (aag) by arginine (agg) at position 48 (B-K48R) and cysteine (tgc) by arginine (cgc) at codon 276 (B-C276R) were found in strains BC1 and V212-2, respectively. Strain R35-3 had a proline (ccc) residue [instead of serine (tcc)] at position 51 in SdhC (C-S51P), and isolate R39-1
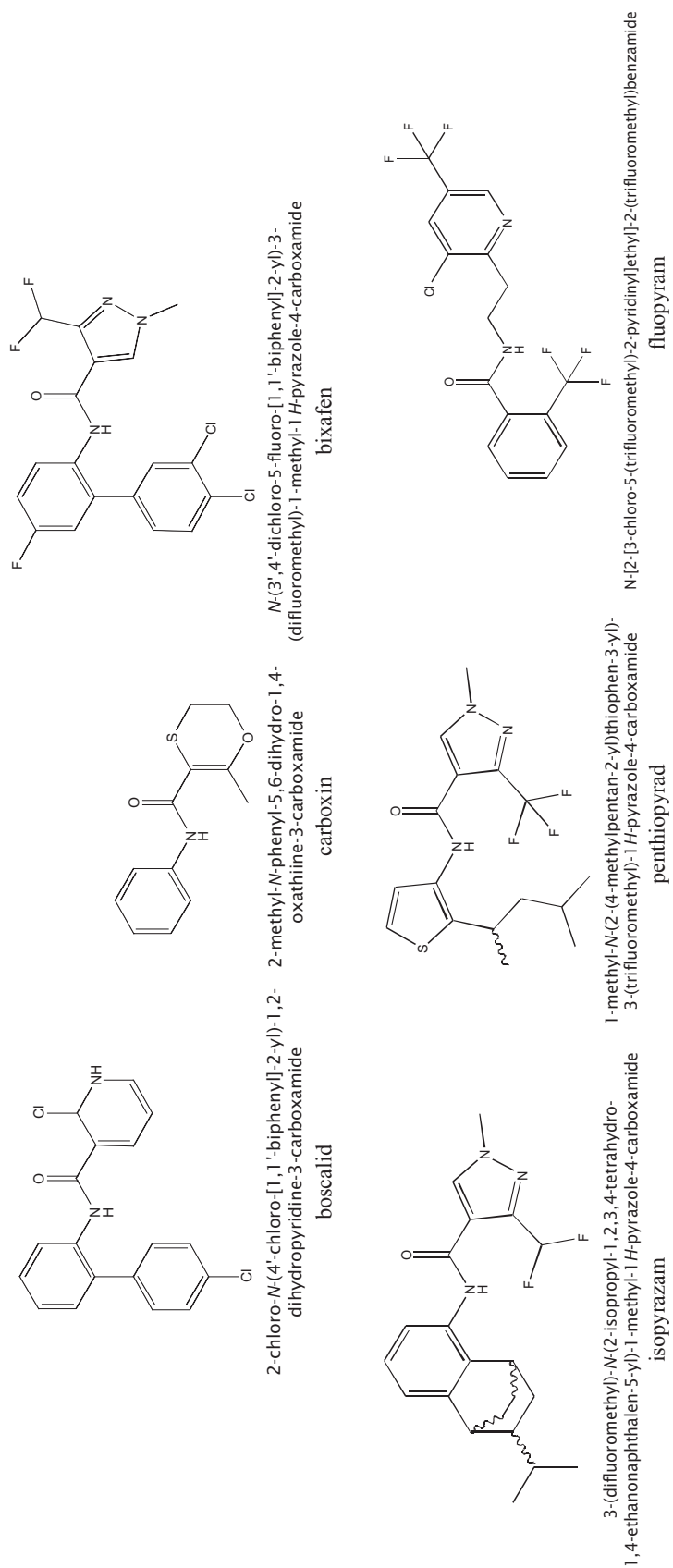
N-(3',4'-dichloro-5-fluoro-[1,1'-biphenyl]-2-yl)-3-
(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide
bixafen

N-[2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]ethyl]-2-(trifluoromethyl)benzamide
fluopyram

2-methyl-N-phenyl-5,6-dihydro-1,4-
oxathiine-3-carboxamide
carboxin

1-methyl-N-(2-(4-methylpentan-2-yl)thiophen-3-yl)-
3-(trifluoromethyl)-1H-pyrazole-4-carboxamide
penthiopyrad

2-chloro-N-(4'-chloro-[1,1'-biphenyl]-2-yl)-1,2-
dihydropyridine-3-carboxamide
boscalid

3-(difluoromethyl)-N-(2-isopropyl-1,2,3,4-tetrahydro-
1,4-ethanonaphthalen-5-yl)-1-methyl-1H-pyrazole-4-carboxamide
isopyrazam

Fig. 1 Structural formulae and systematic names of the studied succinate dehydrogenase inhibitor (SDHI) fungicides.

Table 1 Characterization of *Mycosphaerella graminicola* field strains.

Isolate	CYP51 amino acid alterations*	Origin	Year	Fungicide sensitivity (EC$_{50}$ in μg/mL)†						
				Carboxin	Boscalid	Bixafen	Isopyrazam	Epoxiconazole	Prothioconazole-desthio	Chlorothalonil
IPO323	None	The Netherlands	1981	1.50	0.970	0.273	0.418	<0.0005	0.0018	0.167
IPO88004	None	Ethiopia	1988	1.43	0.208	0.031	0.107	<0.0005	0.0009	0.145
A12/3B/8	None	USA	1988	1.99	0.210	0.055	0.053	<0.0005	0.0015	0.191
IPO90012	None	Mexico	1990s	2.05	0.300	0.086	0.073	<0.0005	0.0013	0.119
IPO001	None	The Netherlands	1991	1.27	0.203	0.054	0.078	<0.0005	0.0015	0.141
RD14	Y137F	UK	1993	0.622	0.271	0.094	0.119	0.0057	0.0060	0.040
ST16	L50S, G460D	UK	1995	1.61	0.603	0.381	0.539	0.0018	0.0023	0.145
IPO92006	L50S, S188N, N513K	Portugal	1990s	3.75	0.615	0.393	0.169	<0.0005	0.0015	0.218
CTRL01-1	Y137F, S524T	UK	2001	3.13	0.438	0.130	0.176	0.0536	0.0042	0.097
LARS15	L50S, I381V, Y459D	UK	2003	1.40	0.433	0.175	0.247	0.0549	0.0105	0.109
G303	L50S, S188N, A379G, I381V, DEL, N513K	UK	2003	2.25	1.14	0.664	0.591	0.0749	0.0134	0.176
IRE13	L50S, V136A, Y461S	Ireland	2003	0.868	0.197	0.030	0.042	0.0268	0.0127	0.069
IRE30	L50S, V136A, Y461S	Ireland	2003	1.75	0.522	0.090	0.130	0.0399	0.0110	0.162
POL27	L50S, I381V, Y461H	Poland	2004	1.27	0.524	0.207	0.397	0.127	0.0144	0.155
NZ75	Y137F	New Zealand	2004	1.20	0.292	0.057	0.074	0.0056	0.0011	0.235
BD04	G460D	Spain	2006	0.711	0.655	0.127	0.435	0.0056	0.0023	0.127
MM20	None	Spain	2006	3.42	0.394	0.383	0.368	<0.0005	0.0018	0.210
V212-2	V136A, S188N, DEL	UK	2006	2.69	0.415	0.215	0.184	0.0712	0.0118	0.141
4439	None	Finland	2007	0.765	0.604	0.312	0.410	<0.0005	0.0006	0.165
T2	D107V, I381V, N513K, S524T	France	2008	0.608	0.446	0.179	0.294	0.0578	0.0063	0.113
NZ11-1	L50S, S188N, N513K	New Zealand	2008	3.56	0.454	0.078	0.130	<0.0005	0.0019	0.212
I43-2	L50S, V136A, Y461S, S524T	Ireland	2009	1.10	0.630	0.391	0.206	0.244	0.0514	0.104
R35-3	L50S, I381V, Y461H	UK	2009	1.71	0.375	0.183	0.279	0.0910	0.0193	0.254
R39-1	L50S, S188N, A379G, I381V, DEL, N513K	UK	2009	5.52	0.795	0.662	1.10	0.278	0.0262	0.325
BC1	L50S, I381V, Y461H	UK	2009	1.64	0.269	0.095	0.210	0.0818	0.0155	0.316
BC4	L50S, I381V, Y461H	UK	2009	1.42	0.472	0.408	0.328	0.151	0.0270	0.329
TAG71-3	L50S, D134G, V136A, Y461S, S524T	UK	2009	2.05	0.161	0.019	0.020	0.453	0.156	0.336
TAG1-9	L50S, V136A, I381V, Y461H, S524T	UK	2010	1.35	0.498	0.271	0.206	1.50	0.180	0.081
TAG74-3	L50S, S188N, I381V, DEL, N513K	UK	2010	1.46	0.305	0.115	0.123	1.05	0.167	0.151
ROS1.40.1	L50S, V136A, I381V, Y461S, S524T	UK	2010	1.24	0.522	0.402	0.130	0.658	0.152	0.146

*DEL indicates deletion of both Y459 and G460.

†Fungicide sensitivity values (EC$_{50}$) are the means of two measurements.

had two changes in this subunit, a valine (gtc) residue [instead of isoleucine (atc)] at codon 29 (C-I29V) and a glycine (ggc) residue [instead of arginine (cgc)] at codon 54 (C-R54G). Two SdhC changes were also found in strain V212-2, with two asparagine (aac) residues replaced by threonine (acc) at codons 33 (C-N33T) and 34 (C-N34T). For SdhD, strain R35-3 carried a glycine (ggt) residue [instead of alanine (gct)] at codon 10 (D-A10G), whereas IRE30 harboured a unique proline (ccg) residue [instead of arginine (cgg)] at codon 47 (D-R47P). *SdhC* was most variable with regard to additional nucleotide changes leading to synonymous substitutions and sequence variation within intron regions (data not shown).

Docking of carboxin and other SDHIs in the quinone binding site of the *M. graminicola* Sdh complex

Docking studies (Figs 2 and 3) showed carboxin positioned in a similar manner as in the crystal structure of the avian Sdh complex (Huang *et al*., 2006). SdhA did not form part of the quinone binding site. SdhB residues P220, S221, W223, W224, H267 and I269 form the major part of the binding pocket. SdhC (L71, W80, S83, A84 and R87) and SdhD (D129 and Y130) form the remainder of the fungicide binding cavity. The docking study also indicates that the binding energy for the carboxin–Sdh complex is relatively high, suggesting relatively poor binding when compared with the

other fungicides (Table 2). This probably reflects the relatively small size of this molecule, reducing extensive protein–fungicide interactions. Boscalid and bixafen contain an additional phenyl ring compared with carboxin, which is reflected in the lower binding energies. The predicted binding energies of carboxin, boscalid, bixafen and isopyrazam (Table 2) are perfectly in line with the respective baseline fungicide sensitivity levels (Table 1). The
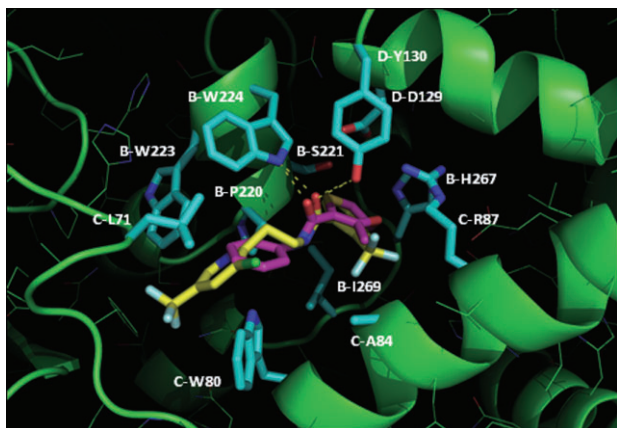

Fig. 2 Docking of carboxin and fluopyram in the quinone binding site. The succinate dehydrogenase (Sdh) structural model is shown with the carbon atoms in green, with the residues forming the binding pocket with the carbon atoms in cyan. Carboxin and fluopyram are shown with the carbon atoms in magenta and yellow, respectively. Hydrogen bonds are indicated as broken lines.

chlorinated phenyl rings of boscalid and bixafen occupy the same binding pocket as the phenyl moiety of docked carboxin (Fig. 3A–C). However, the amide moiety is bound in another position, as is the second phenyl moiety of these fungicides, which is located at the surface of the protein. The pyrazole and pyridine moieties of bixafen and boscalid are found in the same pocket as the nonaromatic ring of carboxin. Binding seems to be stronger for bixafen, suggesting that the pyrazole moiety interacts favourably when compared with the pyridine moiety of boscalid, as both fungicides adopt similar binding modes when docked into the Sdh complex. Docking of isopyrazam (Fig. 3D) and penthiopyrad (Fig. 3E) yields similar binding energies when compared with bixafen (Table 2). However, the binding mode more closely resembles the binding of carboxin. The pyrazole moieties occupy the same pocket as the phenyl group of carboxin, whereas their amide and hydrophobic moieties bind in the same location as the amide and the nonaromatic ring of carboxin. To compare the effectiveness of fluopyram *in silico*, we also docked this novel fungicide. The predicted fluopyram binding is largely similar to the binding of carboxin, as the phenyl ring and the amide moiety are in the same location as the nonaromatic ring and amide of carboxin (Fig. 3F). However, the other moiety of fluopyram, a benzamide group, is separated from the amide bond by two carbons. This facilitates extended binding of this fungicide, as the benzamide moiety is favourably stacked in between two tryptophans, SdhB W223 and SdhC W80 (Fig. 2). There is no such spacer between the amide and the
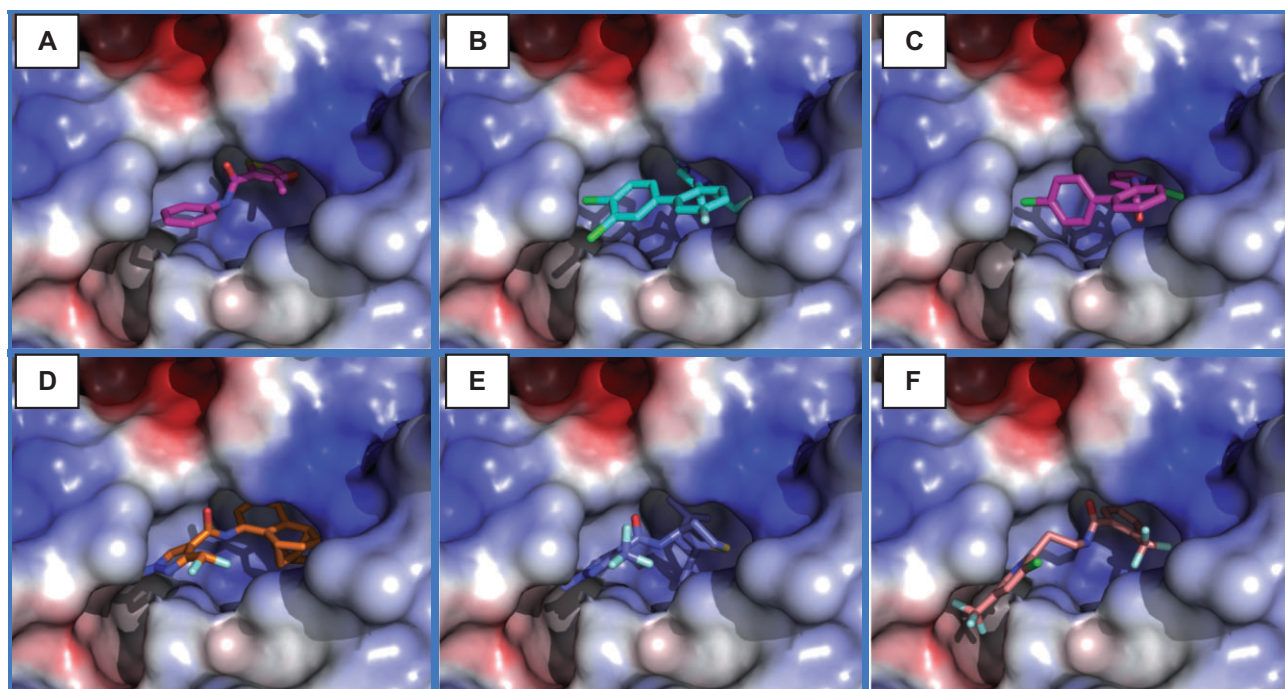

Fig. 3 Binding of succinate dehydrogenase inhibitors (SDHIs) to the quinone binding site of the succinate dehydrogenase (Sdh) complex. Carboxin (A), bixafen (B), boscalid (C), isopyrazam (D), penthiopyrad (E) and fluopyram (F). The electrostatic potential of the protein surface is coloured from red (negative charge) to blue (positive charge).

Table 2 Lowest binding energies obtained by docking of fungicides in a structural model of the wild-type (WT) succinate dehydrogenase (Sdh) complex from *Mycosphaerella graminicola* and fungicide resistance-related Sdh variants.

Inhibitor*	Binding energies for each complex (kJ/mol)†										
	WT	S221P SdhB	R265P SdhB	H267L SdhB	H267Y SdhB	H267N SdhB	I269V SdhB	N86K SdhC	I127V SdhD	D129E SdhD	D129G SdhD
Boscalid	−113.0	−114.9	−114.2	**−111.8**	−112.7	−112.5	−112.8	−114.8	−114.6	−116.1	**−110.5**
Carboxin	−92.0	−92.5	**−90.2**	**−90.0**	−93.1	**−89.1**	**−88.7**	**−90.3**	**−90.5**	−91.1	**−84.0**
Bixafen	−121.9	−129.4	−122.6	−121.8	−122.2	−125.1	**−120.0**	−124.8	−122.7	−121.7	−122.1
Isopyrazam	−122.8	**−115.5**	**−119.8**	**−115.2**	**−113.0**	−126.4	−123.4	**−118.9**	−123.4	**−118.6**	−136.2
Penthiopyrad	−122.4	**−120.5**	−122.2	**−120.2**	**−119.2**	−122.6	**−121.1**	−124.1	−122.2	**−121.1**	−125.6

*The *S*-enantiomer of penthiopyrad and the (C6 *S*, C10 *R*, C21 *S*)-enantiomer of isopyrazam were used for docking studies.
†Higher binding energies in comparison with the wild-type (WT) Sdh variant indicate a weaker binding of the inhibitor to the target. Values with a significant increase (>1 kJ/mol) are presented in bold.

analogous phenyl group in carboxin. This suggests very tight binding for fluopyram, as the binding energy, −138.6 kJ/mol, is the lowest of all the docked SDHIs (Table 2).

Identification of *Sdh* mutations in carboxin-resistant UV mutants

In total 124 mutants, 98 derived from IPO323 (Table 3) and 26 from IRE30 (Table 4), were isolated and characterized. Mutants in the collection had a wide range of nonsynonymous mutations (Fig. 4) and corresponding SDHI sensitivity profiles. Some rare mutants had multiple mutations in the same or in different *Sdh* subunit encoding genes. Figure 4 shows the position of nine key amino acids that were substituted in SDHI-insensitive mutants of *M. graminicola*, and how conserved are these residues in SdhB, SdhC and SdhD sequences from *A. alternata*, *As. oryzae*, *B. cinerea*, *C. cassiicola* and chicken (*Gallus gallus*). SdhB-C137R is unique for *M. graminicola* and no mutations have been found at or in close proximity to this position in SDHI-resistant strains of other fungi. This residue is normally part of an FeS cluster, relatively far from the quinone binding site, and it is expected that such a mutation would result in a nonfunctional protein. SdhB codon S221 (S221P/T) is one position away from codon 225 in *B. cinerea*, where substitutions P225L/F/T have been reported for field strains (Leroux *et al.*, 2010; Stammler *et al.*, 2008). Codon 230 in *B. cinerea*, where N230I has been linked to SDHI resistance (Leroux *et al.*, 2010), is positioned next to W224 in *M. graminicola*, a residue predicted to form part of the quinone binding site (Fig. 2). SdhB codons 265 (R265P), 267 (H267F/L/N/Y) and 269 (I269V) are clustered together, and mutations resulting in H267Y/L/N have also been reported in a range of other fungi (Avenot and Michailides, 2010). SdhC N80K, reported for *Coprinus cinereus* (Ito *et al.*, 2004), is identical to N86K in *M. graminicola*. In addition, SdhC L85P and N86S were also found in *M. graminicola* mutants. SdhC S73P and T90I, reported for *C. cassiicola* (Miyamoto *et al.*, 2010) and *As. oryzae* (Shima *et al.*, 2009), respectively, are within three positions of codons 85 and 86 in *M. graminicola*. SdhD

codons 127 (I127V) and 129 (D129E/G/T) are clustered together. SdhD D89G and D124E, reported in *Paracoccus denitrificans* (Matsson *et al.*, 1998) and *As. oryzae* (Shima *et al.*, 2009), respectively, are equivalent to D129G and D129E in *M. graminicola*. G109V in *C. cassiicola* (Miyamoto *et al.*, 2010), H133R in *A. alternata* (Avenot *et al.*, 2009) and H132R in *B. cinerea* (Leroux *et al.*, 2010) are part of the α-helix that positions the two crucial quinone binding site residues in *M. graminicola*, SdhD D129 and Y130. However, they are too far from the quinone binding site (approximately 12 Å) to have a direct effect on fungicide binding.

Relationship between mutant Sdh variants and SDHI sensitivity

Tables 3 and 4 show the relationship between the different Sdh variants and the sensitivity ranges measured for the different SDHI fungicides. Mutants carrying B-S221P are clustered into three different phenotypic groups depending on the parental strain. In comparison with IRE30 mutants, B-S221P mutants derived from IPO323 have higher levels of resistance to all SDHIs tested, in particular for boscalid with EC$_{50}$ levels above 12.5 μg/mL. For one IPO323-derived mutant, M152, carrying S221P with no other mutations detected in the *SdhB*, *SdhC* and *SdhD* genes, the sensitivity to bixafen and isopyrazam was much lower than for IPO323. For all other Sdh variants tested, there was no clear strain-dependent difference in SDHI sensitivity. Most mutants with multiple mutations in *Sdh* genes (B-P155L/B-H267Y, B-S221P/C-R54G and B-I13V/D-D129E/D-V154A) were more sensitive to most SDHIs tested relative to mutants carrying single amino acid substitutions (B-S221P, B-H267Y and D-D129E). Mutants carrying variant B-H267L were highly resistant to all four SDHIs tested. High levels of boscalid resistance were measured for variants carrying B-C137R, B-S221P (only IPO323-derived mutants), B-H267F, H267Y, C-N86K, D-D129E and D-D129G. Sensitivity levels to bixafen and isopyrazam were similar for mutants carrying different Sdh variants, with the exception of B-I269V and B-S221T

Table 3 Overview of key mutations and associated amino acid substitutions in succinate dehydrogenase (Sdh) complexes of IPO323-derived mutants ($n = 98$) and their succinate dehydrogenase inhibitor (SDHI) sensitivity profiles.

Strains/mutant Sdh variants	Corresponding codon changes	Mutant number	Fungicide sensitivity range (EC$_{50}$ in µg/mL)			
			Carboxin†	Boscalid	Bixafen	Isopyrazam
IPO323			3.50	0.718	0.216	0.316
B-C137R	tgt > cgt	1	159	>12.5	2.01	1.93
B-S221P (M152)*	tcc > ccc	1	11.8	0.344	0.014	<0.001
B-S221P*	tcc > ccc	5	102–190	>12.5	0.761–1.91	0.889–2.34
B-S221T	tcc > act	2	81.7–113	0.959–2.0	0.465–0.583	0.742–1.11
B-R265P	cga > cca	6	133–175	3.26–5.0	0.235–1.02	0.644–1.83
B-H267F	cac > ttc	1	58.1	>12.5	2.2	3.13
B-H267L	cac > ctt/ctc	9	>200	>12.5	>12.5	10.0->12.5
B-H267N	cac > aac	5	81.0–131	0.662–1.33	0.284–0.787	0.451–2.09
B-H267Y	cac > tac	48	67.7–193	>12.5	0.482–3.05	0.232–4.6
B-I269V	att > gtt	7	63.5–143	1.1–2.86	0.397–1.03	1.15–1.86
B-S221P, C-R54G	tcc > ccc, cgc > ggc	1	102	2.65	0.462	1.08
B-H267Y, C-N86S	cac > tac, aac > agc	1	153	>12.5	1.46	2.11
B-D166G, D-D129G	gac > ggc, gac > gga	1	>200	>12.5	4.62	5.13
B-I13V, D-D129E, D-V154A	att > gtt, gac > gaa, gtt > gct	1	40.0	3.08	0.969	1.44
C-L85P	ctc > ccc	1	85.1	1.64	0.792	0.669
C-N86K	aac > aaa	1	112	>12.5	>12.5	>12.5
D-I127V	atc > gtc	1	85.1	1.86	0.433	1.27
D-D129E	gac > gag/gaa	5	85.1–148	11.6->12.5	0.412–2.15	0.666–4.07
D-D129T	gac > acc	1	>200	3.41	3.49	3.6

*Mutants with B-S221P showed two different phenotypes. One mutant derived from IPO323 (M152) was extremely sensitive to bixafen and isopyrazam. The other B-S221P mutants ($n = 5$) were more resistant to all SDHIs tested.

†The carboxin sensitivity reported for strain IPO323 was higher than that reported in Table 4 because of the wider range of fungicide concentrations tested to measure the high resistance levels in the mutants.

Table 4 Overview of key mutations and associated amino acid substitutions in succinate dehydrogenase (Sdh) complexes of IRE30-derived mutants ($n = 26$) and their succinate dehydrogenase inhibitor (SDHI) sensitivity profiles.

Strains/mutant Sdh variants*	Corresponding codon changes	Mutant number	Fungicide sensitivity range (EC$_{50}$ in µg/mL)†			
			Carboxin	Boscalid	Bixafen	Isopyrazam
IRE30			2.82	0.389	0.070	0.088
B-S221P	tcc > ccc	5	57.8–108	0.447–1.09	0.058–0.243	0.096–0.338
B-H267N	cac > aac	4	64.7–99.3	1.11–1.88	0.284–0.81	0.398–2.09
B-H267Y	cac > tac	14	69.0–155	>12.5	0.777–2.6	0.882–4.04
B-P155L, B-H267Y	cca > cta, cac > tac	1	23.6	0.878	0.682	0.486
C-L85P, D-V96A	ctc > ccc, gtc > gcc	1	53.1	3.33	0.874	1.75
D-D129E	gac > gag	1	149	>12.5	1.25	2.4

*Strain IRE30 and related mutants all carry D-R47P in comparison with strain IPO323.

†The carboxin sensitivity reported for strain IRE30 was higher than that reported in Table 4 because of the wider range of fungicide concentrations tested to measure the high resistance levels in the mutants.

mutants that were inhibited more strongly with bixafen than isopyrazam. Interestingly, high levels of resistance to both bixafen and isopyrazam were only measured for mutants carrying B-H267L or C-N86K.

In planta fitness studies on SDHI-resistant UV mutants of *M. graminicola*

A selection of field strains (BC1, BC4 and IRE30) and Sdh mutants derived from IRE30 were further examined with regard to their *in planta* pathogenicity and the ability to produce spores (Table 5). After 21 days, the inoculated areas were completely necrotic and covered with pycnidia for the three field isolates. All mutants produced lesions with pycnidia, but some areas of green leaf tissue were still observed within the inoculated areas. The largest areas of necrosis were observed for mutants M74 and M79 (data not shown). In comparison with IRE30, where 36 ng of pathogen DNA were detected per 50 ng of total DNA after 25 days, the level of pathogen DNA was lower for the mutants, with measured values between 5 and 30 ng. However, half of the mutants tested produced more pycnidiospores than IRE30 after 21 days. The amount of spores harvested per leaf after 21 days was at least five-fold higher than the initial amount of spores inoculated for all mutants and strains tested. Although some small differences in

```
SdhB                *
Mg 131-GVNTLACLCRIPT-143
Aa 141-GVNTLACLCRIPT-153
Ao 113-GVNTLACLCRIPT-125
Bc 136-GVNTLACLCRIPR-148
Cc 140-GVNTLACLCRIPT-152
Gg 117-GGNTLACTKKIDP-129


SdhB                  **   *
Mg 214-CCSTSCPSYWWNS-226
Aa 224-CCSTSCPSYWWNQ-236
Ao 196-CCSTSCPSYWWNS-208
Bc 219-CCSTSCPSYWWNQ-231
Cc 225-CCSTSCPSYWWNQ-237
Gg 201-CCSTSCPSYWWNG-213


SdhB               *  *  *
Mg 261-MSLYRCHTILNCS-273
Aa 271-MSLYRCHTILNCT-283
Ao 243-MSLYRCHTILNCT-255
Bc 266-MSLYRCHTILNCT-278
Cc 272-MSMYRCHTILNCS-284
Gg 248-FSLYRCHTIMNCT-260


SdhC              ***  *
Mg 081-YLSALNRVTGVAA-093
Aa 070-YASSLNRITGITL-082
Ao 082-IGSSFHRITGFAL-094
Bc 081-IMSGLNRITGCIL-093
Cc 070-YASSFNRITGVAL-082
Gg 037-AMSITHRGTGVAL-049


SdhD      *     *    * *
Mg 118-HSYIGFQSAITDY-130
Aa 133-HSHIGFESCIIDY-145
Ao 113-HSHIGFQAAIIDY-125
Bc 132-HSHIGFESCITDY-144
Cc 105-HSHIGFEACVIDY-117
Gg 100-HGHWGLGQVITDY-112
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Fig. 4 Positions of key amino acid alterations in different succinate dehydrogenase (Sdh) subunits from succinate dehydrogenase inhibitor (SDHI)-resistant mutants of *Mycosphaerella graminicola*. Partial Sdh subunit sequences are presented for *M. graminicola* (Mg), *Alternaria alternata* (Aa), *Aspergillus oryzae* (Ao), *Botrytis cinerea* (Bc), *Corynespora cassiicola* (Cc) and *Gallus gallus* (Gg). Positions of key amino acid residues are marked with a star. Shaded residues are amino acid residues that have been altered and linked with SDHI resistance. Bold amino acids are conserved residues.

symptom expression, fungal growth and spore production were measured for individual mutants, these differences were not linked to a particular Sdh variant (e.g. B-S221P, B-H267N, B-H267Y and D-D129E).

DISCUSSION

With the exception of B-C137R, the resistance-related amino acid substitutions discovered in the mutant library are all positioned around the quinone binding pocket of the Sdh complex of *M. graminicola* formed by subunits B, C and D. Most (e.g.

B-S221P/T, B-H267F/L/N/Y, B-I269V and D-D129E/G/T) are directly involved in forming the cavity in which SDHI fungicides bind. Docked carboxin (Fig. 2) is positioned in a similar manner as found in the crystal structure of the avian Sdh complex (Huang *et al*., 2006). This confirms that the docking procedure provides reasonable binding mode predictions. Based on the binding energies, the model predicts that bixafen and isopyrazam will bind more strongly than boscalid, whereas carboxin will be less effective. The model can also predict the binding efficacies of SDHI fungicides to mutated Sdh variants (Table 2). The prediction that B-H267L will be resistant to most SDHIs tested was in line with fungicide sensitivity testing (Table 3). Docking studies also demonstrated a different binding for fluopyram with the Sdh complex of *M. graminicola* (Figs 2 and 3F), a finding consistent with the absence of cross-resistance to fluopyram in highly boscalid-resistant isolates of *A. alternata* (Avenot and Michailides, 2010), *C. cassiicola* and *Podosphaera xanthii* (Ishii *et al*., 2011). However, the model was not always consistent with biological data, in particular for B-H267Y, where stronger binding was predicted for carboxin and bixafen. In addition, the predicted binding modes for isopyrazam and penthiopyrad were unexpected because the orientation of the amide bond was found to be maintained across carboxamides in docking studies performed at Syngenta (G. Scalliet, personal communication). For isopyrazam, the pyrazole ring was predicted to be oriented towards the bottom of the quinone binding site. The docking simulations used in this study were performed without any bias towards a known binding mode of related fungicides. Further refinement of the model, taking into account residues at longer distances and specific interactions between inhibitors and key residues, will further improve the predictive power.

The diversity and high frequency of particular resistant mutants generated by UV mutagenesis demonstrate a potentially high risk for resistance development in *M. graminicola* to SDHIs. In total, 16 key amino acid substitutions conferring resistance to SDHIs were identified at five different positions in SdhB (137, 221, 265, 267 and 269) and two each in SdhC (85 and 86) and SdhD (127 and 129). In addition, several other amino acid substitutions (B-I13V, B-P155L, B-D166G, C-R54G, D-V96A and D-V154A) were found in combination with these 16, with most contributing to a more sensitive phenotype. Sixty-four of the 124 mutants isolated and characterized carried B-H267Y. Because B-H267Y variants have lower carboxin resistance than B-H267L variants, only nine of which were isolated, a higher fitness cost for B-H267L variants seems likely. Shima *et al*. (2009) reported different levels of succinate-cytochrome *c* reductase activity from crude mitochondrial fractions of wild-type and carboxin-resistant UV mutants of *As. oryzae*, including counterparts of the *M. graminicola* B-H267Y, B-H267L, B-H276N and D-D129E variants. The basal SDH activity of all mutants was significantly lower than that of the wild-type, with the lowest relative activity measured for B-H267L (18%) and

Table 5 *In planta* fitness studies on field strains and succinate dehydrogenase inhibitor (SDHI)-resistant laboratory mutants of *Mycosphaerella graminicola*.

Strains/mutants*	SDHI-resistant Sdh alterations†	Amount of DNA (pg)‡				Amount of spores§
		7 days	14 days	21 days	25 days	
BC1		43 ± 43	2544 ± 17	6 047 ± 1 668	32 270 ± 1 750	3.67E + 05
BC4		334 ± 50	1625 ± 774	7 419 ± 1 663	29 010 ± 2 000	3.58E + 05
IRE30		184 ± 44	100 ± 1.8	17 135 ± 615	36 245 ± 1 495	3.00E + 05
M77	B-S221P	248 ± 49	1658 ± 487	1 968 ± 114	15 330 ± 360	3.33E + 05
M99	B-S221P	57 ± 12	148 ± 15	10 749 ± 791	17 115 ± 145	2.67E + 05
M125	B-S221P	169 ± 0.5	100 ± 29	4 556 ± 941	5 334 ± 2 168	2.17E + 05
M73	B-H267N	281 ± 7.6	73.4 ± 2.3	12 140 ± 860	21 045 ± 2 015	5.67E + 05
M126	B-H267N	143 ± 16	229 ± 48	3 174 ± 205	14 630 ± 440	6.50E + 05
M74	B-H267Y	290 ± 45	1482 ± 74	12 500 ± 890	16 115 ± 275	4.17E + 05
M79	B-H267Y	252 ± 31	419 ± 104	8 086 ± 81	29 770 ± 3 310	2.92E + 05
M80	B-H267Y	63 ± 6.7	189 ± 31	9 490 ± 2 820	12 384 ± 2 787	1.42E + 05
M121	B-H267Y	256 ± 39	572 ± 3.7	4 641 ± 15	9 479 ± 1 251	4.42E + 05
M81	D-D129E	159 ± 22	344 ± 52	6 831 ± 812	24 370 ± 0	1.33E + 05

*Mutants (M numbers) are all derived from strain IRE30.

†Succinate dehydrogenase (Sdh) alterations B-K48R in strain BC1 and D-R47P in IRE30 are not associated with SDHI resistance.

‡Amount of DNA presented is per 50 ng of total DNA extracted from inoculated 2-cm segments of four leaves. Mean values of two measurements ± standard errors are given.

§Amount of spores is the mean amount of spores collected per leaf sampled 21 days after inoculation.

the highest for D-D129E (56%). In the presence of 100 μM carboxin, the basal SDH activity of B-H267L was fully retained, whereas the basal activity of D-D129E was reduced to 33%. The isolate-dependent SDHI sensitivity phenotype with B-S221P mutants cannot be explained by differences in the amino acid sequences of the SdhA, SdhB and SdhC subunits. In comparison with IPO323, only one residue is different in IRE30 (D-R47P). It is likely that other changes in the mitochondrial electron transport chain are responsible. IRE30 is unique because it carries cytochrome *b* F129L alleles which, in comparison with G143A alleles, confer intermediate levels of resistance to QoI fungicides (Lucas and Fraaije, 2008). Fisher *et al*. (2004) showed that modified cytochrome *b* quinone outside sites can affect cytochrome *c* reductase activity using yeast cytochrome *b* as a model. The combination of SdhB S221P and cytochrome *b* F129L might have a more deleterious effect on fitness than other combinations of Sdh variants and cytochrome *b* alleles. Increased *in vitro* sensitivity to boscalid has also been reported for QoI reduced-sensitive field strains of *Alternaria solani* carrying cytochrome *b* F129L alleles (Pasche *et al*., 2005) and QoI-resistant laboratory mutants of *B. cinerea* (Markoglou *et al*., 2006). One IPO323-derived mutant (M152) was less resistant to carboxin than other mutants carrying B-S221P, and showed increased sensitivity to bixafen and isopyrazam. For this mutant, additional mutations causing changes in other components of the mitochondrial electron transport chain cannot be excluded.

The field strains were all well inhibited *in vitro* with the multi-site inhibitor chlorothalonil, with EC$_{50}$ recorded between 0.040 and 0.336 μg/mL. Ranges for the SDHIs were tight, but some strains, G303 and R39-1, in particular, showed slightly increased EC$_{50}$ values for one or more SDHIs. Sequencing revealed that strain R39-1 carried two unique alterations in SdhC, I29V and R54G. These residues are too far (>25 Å) from the quinone binding site to have a direct effect on fungicide binding. However, C-R54G was also found in combination with B-S221P in one mutant and increased the sensitivity to boscalid and bixafen, in comparison with most IPO323-derived mutants carrying B-S221P alone (Table 3). Intensive monitoring since 2003 of *M. graminicola* field strains for SDHI sensitivity by agrochemical companies has shown a fully sensitive picture (FRAC SDHI Working Group, unpublished). A wide range of EC$_{50}$ values were measured for epoxiconazole (<0.0005 to 1.5 μg/mL) and prothioconazole-desthio (0.0006–0.180 μg/mL). Prothioconazole-desthio is approximately 100-fold more active than prothioconazole (B. A. Fraaije, unpublished), and *in planta* and/or *in vivo* metabolism of prothioconazole by the fungus itself has been suggested to contribute to the inhibition of CYP51 (Parker *et al*., 2011). Increased sensitivity to azoles, both *in vitro* and *in planta* (Fraaije *et al*., 2011), has been directly linked to changes in the CYP51 target protein, and novel CYP51 variants are continuously evolving (Brunner *et al*., 2008; Cools and Fraaije, 2008; Cools *et al*., 2011; Leroux and Walker, 2011). There is a concern that the activity of the most active azoles might erode to such an extent that field performance will be compromised, as reported for some older azoles (Clark, 2006). The use of mixtures as a resistance management strategy will only work with effective noncross-resistant fungicides acting simultaneously at key fungal developmental stages. Further monitoring of sensitivity shifts to both SDHI and DMI fungicides is required, and an improved understanding of the underlying resistance mechanisms will lead to the exploitation of the chemical diversity within these fungicide classes.

Although these laboratory studies cannot provide direct evidence of the potential for field resistance, the knowledge arising

from this investigation will enable the prediction of which mutations are most likely to be selected in field populations by different SDHIs, and also aid in the development of DNA-based diagnostics based on allele-specific PCR. In addition to the sequence information on Sdh complexes in field strains of *M. graminicola*, different mutations conferring resistance to SDHIs were identified. The early detection of resistant alleles at low frequencies, coupled with risk evaluation, will allow the implementation of anti-resistance strategies to prolong the lifetime and cost-effectiveness of this class of fungicides.

EXPERIMENTAL PROCEDURES

Isolation of fungal strains

Field population sampling and *M. graminicola* isolation from plants were performed as described by Fraaije *et al*. (2007). Conidial suspensions were streaked onto yeast extract peptone dextrose agar (YEPD agar; ForMedium, Norwich, Norfolk, UK) amended with penicillin G sodium and streptomycin sulphate at 100 μg/mL, and incubated for 5 days at 23 °C. Single colonies subsequently grew in the form of yeast-like budding cells. Single spore strains were propagated by transferring single colonies to fresh YEPD agar plates. Strains were either used directly in sensitivity assays or stored in 80% glycerol at –80 °C. All strains used in this study are listed in Table 1.

Generation of SDHI-resistant UV mutants

Ten thousand spores of *M. graminicola* strains IPO323 or IRE30 were plated out onto YEPD agar amended with carboxin at 50 μg/mL. Plates were exposed to UV light at 500 or 600 J/m² using a Stratagene UV cross-linker (model 1800; Stratagene Corporation, La Jolla, CA, USA), which resulted in approximately 20% survival. Putative carboxin-resistant mutants were isolated from growing colonies after 14 days of incubation at 21 °C in the dark. These mutants were grown for another generation on carboxin-amended YEPD agar, harvested in sterile distilled water and stored in 80% (v/v) glycerol at –80 °C for further use.

Isolation of fungal genomic DNA, PCR and DNA sequencing

Genomic DNA for PCR was extracted and quantified according to the protocol of Rudd *et al*. (2010). To amplify genes encoding for SdhB, SdhC and SdhD, specific primers for each subunit (see Table 6) were designed based on the available genome sequence for strain IPO323 downloaded from the Joint Genome Institute (JGI) *M. graminicola* genome website (URL: http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html). PCR was carried out in an Applied Biosystems (Foster City, CA, USA) Veriti 96-well thermal

Table 6 Polymerase chain reaction (PCR) primers to amplify succinate dehydrogenase (*Sdh*) genes from *Mycosphaerella graminicola*.

Primers	Target	Sequence (5′–3′)	Amplicon size (bp)
Mgsdhbf1	*SdhB*	ACTCTTCTCACATACCACACA	1051
Mgsdhbr1		CTTTCCAATCATCTCGTTCCAT	
Mgsdhcf1	*SdhC*	GGCACATCGCGTCTCACG	736
Mgsdhcr1		TCCCTCATTTCCCCATCCAC	
Mgsdhdf1	*SdhD*	CTCACCCTCACCGTCGCC	715
Mgsdhdr1		CCATCTACAACTTCTGCTCAATC	

cycler in a final volume of 30 μL containing 2.5 units of Easy-A high-fidelity PCR cloning enzyme (Stratagene Corporation), 3 μL of 10 × Easy-A reaction buffer, 200 μM of each deoxynucleoside triphosphate (dNTP), 1.0 μM of each primer and 30 ng of fungal template DNA. Amplification conditions consisted of 94 °C for 2 min, followed by 35 cycles at 94 °C for 20 s, 57 °C (SdhB), 66 °C (SdhC) or 58 °C (SdhD) for 30 s, and 72 °C for 2 min, with a final DNA extension at 72 °C for 8 min. PCR products were separated in ethidium bromide-stained 1% agarose gels run in 1 × tris(hydroxymethyl)aminomethane (Tris)–borate–ethylenediaminetetraacetic acid (EDTA) buffer and exposed to UV light to visualize DNA fragments. PCR products were cloned into pGEM-T easy vector (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions, and subsequently sequenced with M13 sequence primers using a dideoxy chain termination reaction method. Sequences were assembled and further aligned with Vector NTI and CLUSTALW software.

Phenotyping and genotyping of carboxin-resistant UV mutants and field strains

Wells of flat-bottomed microtitre plates (TPP92696, Techno Plastic Products, Trasadingen, Switzerland) were filled with 100 μL of double-strength Sabouraud dextrose liquid medium (SDLM; Oxoid, Basingstoke, Hampshire, UK) without fungicide or amended with different fungicide concentrations. The following concentrations were used to test field strains: bixafen, boscalid and isopyrazam (0.00023, 0.00092, 0.0037, 0.0146, 0.0586, 0.234, 0.938, 1.875, 3.75, 7.5 and 15 μg/mL), carboxin (0.0977, 0.195, 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 μg/mL), chlorothalonil (0.0195, 0.0391, 0.0781, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10 and 20 μg/mL), epoxiconazole (0.00014, 0.00051, 0.00192, 0.0072, 0.0270, 0.101, 0.379, 1.42, 5.33, 20 and 75 μg/mL) and prothioconazole-desthio (Parker *et al*., 2011), a metabolite of prothioconazole (0.00034, 0.0010, 0.0030, 0.0091, 0.0274, 0.0823, 0.247, 0.741, 2.22, 6.67 and 20 μg/mL). For the mutants, higher concentrations of SDHIs were used: bixafen, boscalid and isopyrazam (0.00076, 0.0031, 0.0122, 0.0488, 0.195, 0.781, 3.13, 12.5 and 25 μg/mL) and carboxin (0.488, 0.977, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250 and 500 μg/mL). Before dilution in media, chemicals were dissolved in acetone, with the exception

of chlorothalonil, which was dissolved in dimethylsulphoxide (DMSO). One hundred microlitres of spore suspension (2.5×10^4 spores/mL) of *M. graminicola* strains or UV mutants were added to each well. Plates were incubated for 4 days at 23 °C, and growth was measured at 630 nm using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany) in well-scanning mode with a 2×2 matrix of scanning points within a 3-mm diameter. Fungicide sensitivities were determined as the 50% effective concentration to inhibit growth (EC_{50} in µg/mL) using a dose–response relationship according to OPTIMA software. Based on the phenotyping results, the *SdhB*, *SdhC* and *SdhD* genes of mutants with differences in SDHI sensitivity were sequenced. Sequence analysis identified putative resistance-conferring mutations. Subsequently, pyrosequencing assays were developed to screen the remaining mutants for the presence of these mutations. The *SdhB*, *SdhC* and *SdhD* genes of mutants testing negative by pyrosequencing for the identified mutations were also sequenced. This was repeated until all mutants were genotyped.

Pyrosequencing assays

Primers (forward primer, reverse primer and sequence primer) were designed with Pyrosequencing Assay Design Software (Version 1.0.6; Biotage, Uppsala, Sweden) targeting different regions of *SdhB*, *SdhC* and *SdhD* in which SDHI resistance-conferring mutations were identified (Table 7). Polymerase chain reactions (PCRs) were carried out in a Biometra T3 thermocycler (Biotron, Göttingen, Germany) with 0.2 units of Red Hot DNA polymerase (ABgene, Epsom, Surrey, UK) using 20 mM $(NH_4)SO_4$, 75 mM Tris-HCl (pH 9.0), 0.01% Tween-20, 1.5 mM $MgCl_2$, 150 µM of each dNTP, 0.5 µM of each primer and 20 ng template DNA in a final volume of 20 µL. Cycling conditions were as follows: 94 °C for 2 min, followed by 40 cycles at 94 °C for 10 s, 61 °C (SdhB codon 221) or 65 °C (other targets) for 20 s and 72 °C for 30 s, with a final DNA extension at 72 °C for 5 min. Single-stranded biotinylated PCR products were prepared using the Pyrosequencing Vacuum Prep Tool (Biotage). Three microlitres of Streptavidin

Table 7 Pyrosequencing assay to detect mutations in *SdhB* and *SdhD*.

Target	Amino acid substitutions	Primer sets*
SdhB	S221P, S221T	F: CATTCTCTGCGCATGCTG* R: CGTTGATCCATCGGTATGACT S: CCGAGTTCCACCAGT
SdhB	R265P, H267F, H267L, H267N, H267Y, I269V	F: CGCAAGGACGCACTCAACA* R: GGGCAGGTCCTTGAGCAATT S: GTCCTTGAGCAATTCAG
SdhD	I127V, D129E, D129G, D129T	F: CCCACCCGTATCATTCTGACA* R: GAGCTTGCGGGTTTTGGG S: CGCCATGAGGGGAAG

*Symbols F, R and S indicate forward, reverse and sequence primers, respectively; primers with 5′ biotin label are marked. All assays were carried out with the nucleotide dispensation order 10 (CTAG) on the PyroMark Q96ID.

Sepharose HP beads (GE Healthcare, Uppsala. Sweden) were added to 40 µL of binding buffer (10 mM Tris-HCl, pH 7.6, 2 M NaCl, 1 mM EDTA, 0.1% Tween-20) and mixed with 15 µL of PCR product and 25 µL of sterile distilled water for 10 min at room temperature using an Orbis plate shaker (Mikura, Lower Beeding, West Sussex, UK). Beads containing the captured DNA product were aspirated onto filters after applying a vacuum, washed with 70% (v/v) ethanol for 5 s, rendered single stranded with denaturation solution (0.2 M NaOH) for 10 s and neutralized with washing buffer (10 mM Tris-acetate, pH 7.6) for 5 s. The vacuum was released and beads with biotinylated single-stranded DNA were transferred into a PSQ 96-well plate (Biotage) containing 45 µL of annealing buffer (20 mM Tris-acetate, 2 mM magnesium acetate, pH 7.6) and 0.5 mM sequence primer. Pyrosequencing reactions were performed and analysed on a PyroMark Q96ID according to the manufacturer's instructions using a PyroMark Gold Q96 QSA Reagent Kit (Biotage), and the nucleotide dispensation orders are shown in Table 7.

In silico studies on the binding of SDHIs to the Sdh complex of *M. graminicola*

For docking studies of fungicides in the *M. graminicola* Sdh complex, the crystallographic coordinates of the avian Sdh complex were taken from the Protein Data Bank (PDB). The Sdh complex of chicken (PDB code 2FBW) is similar to that of *M. graminicola* (70% sequence identity) and also contains a bound carboxin molecule. By replacing all residues within a 14-Å radius of the bound carboxin with the corresponding residues of the *M. graminicola* Sdh complex using PyMOL, a structural model of the fungal quinone binding site was prepared. All fungicides tested were also prepared in PyMOL. The Molegro Virtual Docker (MVD; Molegro ApS, Aarhus, Denmark) was used for docking simulations. For docking simulations, the structural model and all fungicides were first subjected to energy minimization in MVD. For each resistance-related mutation in one of the subunits, the corresponding mutant protein was built and optimized. Fungicides were docked within a sphere (radius, 12 Å) that encompassed the quinone binding site. For docking, the default settings were used, except for the grid resolution which was set to 0.2, and a total of 25 runs was performed with a maximum of 500 iterations. The best superimposed positions with lowest MolDock energy scores were used in this study. For the visualization of the docked fungicides, PyMOL was used.

In planta fitness studies on Sdh mutants and field strains of *M. graminicola*

In planta pathogenicity tests were carried out on 2–3-week-old wheat seedlings of cultivar Riband according to Keon *et al.* (2007). In total, 18 leaves per strain were inoculated with spore suspensions [2.5×10^5 spores/mL in 0.1% (v/v) Tween-20] using a mini

'mist' sprayer which covered up to a 4-cm length of the leaf (approximately 100 μL of inoculum per leaf segment). After 72 h of incubation at 100% relative humidity in polystyrene boxes, inoculated plants were incubated at 16 °C with a 16-h light period at 88% relative humidity for up to 25 days to allow symptoms to develop. Visual assessments, recording the extent of pycnidia formation and chlorosis, were conducted 21 days after inoculation. DNA was also extracted from four 2-cm inoculated leaf segments at several time points (7, 14, 21 and 25 days after inoculation) and the amount of pathogen DNA was quantified in 50-ng DNA samples according to Fraaije *et al.* (2005) using the CY5 probe to quantify the presence of the cytochrome *b* gene of *M. graminicola*. Spores were also counted from three inoculated leaves after 21 days. Three 2-cm leaf segments were added to 2-mL tubes and incubated overnight in the dark at room temperature in the presence of 300 μL of 0.2% Tween-20 solution. Spores were harvested after two rounds of spinning and shaking at 4.0 m/s in a FastPrep machine (Savants Instruments, Holbrook, NY, USA). The amount of spores collected was determined microscopically using a haemocytometer.

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