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Fungicide Resistance: are we winning the battle but losing the war?

Resistance to fungicides in French populations of *Septoria tritici*, the causal agent of wheat leaf blotch

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Summary

In field strains of *Septoria tritici*, resistance to benzimidazoles, strobilurins and inhibitors of sterol 14α-demethylation (DMIs) is determined by mutations in genes encoding their respective target sites. For benzimidazoles and strobilurins, resistance levels are high and result in a reduced field performance of these fungicides. Regarding DMIs, several mutations have been identified and determine low to medium resistance levels; moreover, cross resistance between DMIs is not always observed. The practical efficacy of DMIs, especially triazoles, remains good. Under field conditions, the highest selection pressures towards strains highly resistant to QoIs and moderately resistant to DMIs are recorded when respectively strobilurins and triazoles are applied at high doses. The combinations of members of group with reduced doses have a generally a less pronounced effect. Prochloraz was the only DMI did not select strains moderately resistant to this class of fungicides.

Key-words: *Septoria tritici*, wheat leaf blotch, resistance, benzimidazole, strobilurine, triazole, QoI, DMI

Introduction

Septoria tritici, the causal agent of leaf blotch, is the major foliar disease of wheat in France and many other European countries. The crop damage during summer is generally attributable to repeated cycles of the asexual stage of this fungus, in which pycnidia give rise to rain splash-dispersed pycnidiospores. This causes, the disease to move vertically within the canopy. The sexual stage *Mycosphaerella graminicola* plays a role in the disease cycle with ascospores often regarded as the primary source of inoculum; recent studies conducted in the UK suggest that these ascospores are be released throughout the year, being dispersed in air currents rather by water-splash (Hunter *et al.*, 1999).

Control of *Septoria tritici* primarily with the use of fungicides applied one to three times between growth stages GSZ32 and GSZ69. In France, the key treatment has to be done between GSZ37 and GSZ45 (Le Hénaff *et al.*, 2004).

Several families of fungicides can be used against *S. tritici*. The oldest ones are multi-site inhibitors (e.g. chlorothalonil, folpet, mancozeb), whose preventive activity is related to inhibition of spore germination. This effect is related to the blocking of several enzymes involved in the respiratory process. A second group includes carbendazim and also the related activities benomyl

and thiophanate-methyl, which were the first systemic fungicides introduced on wheat in the early 1970s. These benzimidazoles derivatives, which bind to β-tubulin (one of the major protein of cellular microtubules) prevent the growth of fungi and consequently can exhibit curative activities. As for DMIs (inhibitors of sterol α-demethylation), they have been the main family of fungicides used against *S. tritici* over the last 20 years. Within this class of SBIs (Sterol Biosynthesis Inhibitors), the registered active ingredients are triazole derivatives (e.g. epoxiconazole, fluquinconazole, tebuconazole). The majority are systemic and exhibit curative activities against many fungi including *S. tritici.* Since 1997, strobilurins (e.g. azoxystrobine, kresoxim-methyl, picoxystrobin, pyraclostrobine, trifloxystrobin) have become a key component for the control of *S. tritici* in France they are generally used in mixture with DMIs. These strobilurins, as well as the oxazolidinedione famoxadone, are specific inhibitors of the mitochondrial complex III. They are classified as QoI inhibitors, because they bind to the quinol oxidation site of cytochrome b, one of the major components of complex III. In the field, these specific respiratory inhibitors exhibit a long lasting activity although the best efficacies are recorded when they are applied preventively.

The monitoring conducted in France since the introduction of QoIs (1997) has allowed us to characterise strains resistant towards the respiratory inhibitors and also to DMIs and benzimidazoles. Their distribution within French populations and also the impact of treatments will be documented.

Material and Methods

Field samples and sampling preparation

Samples consisted of 20 to 30 leaves randomly collected in a field or a plot. Leaf fragments bearing lesions were dipped in sterile water to collect pycnidiospores. To get pure cultures, cirri emerging pycnidia were picked and transferred onto MYA plates (20 g malt, 5 g yeast extract, 12.5 agar L^{-1}) amended with penicillin and streptomycin (50 mg.l-1 each). After an incubation time of 3 to 4 days at 19°C, single colonies were transferred onto fresh MYA plates. These isolates produced sporidia (yeast-like cells) that were used in fungicide sensitivity assays.

Biological tests

The action of fungicides upon germ-tube elongation was studied by spreading sporidia or pycnidiospores onto the surface of an agar medium (10 g glucose, 2 g K_2HPO_4, 2 g KH_2PO_4, 12.5 g agar L^{-1}) amended with fungicides. All tested compounds were technical grade and stock solutions were prepared in ethanol-; the final concentration of ethanol in agar media never exceeded 0.5%. After an incubation time of 48 h at 19°C, in the dark, the lengths of apical germ tubes were estimated under a microscope (Leroux *et al.*, 2000).

Towards individual strains of *S. tritici,* For each fungicide, a range of concentrations was tested on individual strains of *S. tritici.* The dose-response curve then allowed the concentration causing a 50% reduction in germ-tube elongation (EC_{50}) to be determined. Resistance levels (RLs) were estimated as ratios of EC_{50} resistant phenotype / EC_{50} sensitive phenotype.

When testing populations of *S. tritici,* a bulk suspension of pycnidiospores was tested towards discriminating concentrations of fungicides. For each condition, the percentage of the resistant phenotype was estimated by counting spores exhibiting long germ-tubes (Leroux *et al.*, 2005*a,b*).

Molecular tests

A CYP51 gene fragment was obtained for various strains of *S. tritici* either sensitive or resistant to DMIs by using the following primers: A sense 5'/3' (CAC TCT TCA TCT GCG ACC GAG TC) and B reverse 5'/3' (CT GCT GTA ATC CGT ACC CAC CAC). They correspond respectively to the amino-acid sequences HSSSATE (codons 314-320) and VVGTDYS (codons 514-520). This choice was determined by the fact that Cools *et al.* (2005*a,b*) previously proposed that several

mutations between codons 350 and 520 determined DMI-resistance.

A collaboration with Syngenta also allowed for the comparison the frequency of QoI resistance within populations of *S. tritici* by using our bioassay or quantitative real-time PCR (Q-PCR) which determined the single nucleotide polymorphism in the cytochrome b gene leading to the G143A substitution (Gisi *et al.*, 2005).

Results

Resistance to benzimidazoles

When treated by carbendazim, the sporidia of wild type strains were inhibited at low concentrations (EC50 values about 0.005 mg^{-1}). In contrast, all benzimidazole resistant strains collected in the field were highly resistant to carbendazim (RLs greater than 200) and also to the other benzimidazoles. This qualitative resistance was always associated with an increased sensitivity to the phenylcarbamate diethofencarb (Leroux *et al.*, 2005*a,b*). As observed in several other fungi, this negative cross resistance between benzimidazoles and phenylcarbamates was determined by a single base pair mutation in codon 198 of the gene encoding β-tubulin, leading to a change from glutamate (E) to alanine (A).

The monitoring of field populations of *S. tritici* conducted with carbendazim and diethofencarb (5 mg L^{-1}) indicated that benzimidazole resistance is generalised in France. Indeed, more than 90% of the tested populations exhibited frequencies of benzimidazole-resistant strains higher than 50% (Table 1). This situation indicates that benzimidazole-resistant strains can persist in the absence of treatments by this class of anti-microtubule fungicides.

Table 1. *Evolution of resistance to benzimidazoles (BenR), DMIs (TriR) and QoIs (StrR) in France*

Years	Number of samples	Percentage of samples with resistant strains				
		> 50% BenR	> 50% TriR	StrR strains		
				ND[a]	≤ 50%	> 50%
1997	70	99	99	100	0	0
1998	154	95	100	100	0	0
1999	161	91	100	100	0	0
2000	146	90	100	100	0	0
2001	89	98	98	100	0	0
2002	111	91	100	100	0	0
2003	118	95	98	44	42	14
2004	467	92	100	18	44	38
2005	740	98	97	6	28	66

[a]: ND : Not Detected

Resistance to QoIs

For this class of QoIs, in *S. tritici*, the situation is similar to that observed with benzimidazole fungicides. Indeed, wild-type strains are inhibited by low concentrations of strobilurins (EC50 values respectively of 0.008 and 0.0002 mg L^{-1} for azoxystrobin and pyraclostrobin) whereas resistant strains (StrR) exhibit high resistant levels towards this class of respiratory inhibitors (RLs respectively of 250 and 1500 for azoxystrobin and pyraclostrobin). In addition, cross-resistance affects concerns famoxadone, another member of QoI fungicides but not other respiratory inhibitors, such as boscalid or multisite fungicides (e.g. chlorothalonil) (Table 2). As observed in several other fungi, this resistance is determined by a point mutation in the cytochrome b gene resulting in the substitution of glycine (G) by alanine (A) at position 143 (Gisi *et al.*, 2005). It is

therefore easy to determine the structure of fungal populations by using molecular or biological tests.

Table 2. In-vitro *effects of respiratory inhibitors towards field strains sensitive (StrS) or resistant (StrR) to QoIs*

Fungicides	EC_{50} $mg.l^{-1}$ [a] for wild type strains (StrS)	Resistance levels[b] in StrR strains
azoxystrobin	0.008	250
kresoxim-methyl	0.008	>1250
picoxystrobin	0.002	1000
pyraclostrobin	0.0002	1500
trifloxystrobin	0.0002	2000
famoxadone	0.01	200
boscalid	0.15	1.0
chlorothalonil	0.1	1.0

[a] EC_{50} values towards germ-tube elongation (see material and methods)
[b] Resistance levels or RLs : EC_{50} StrR strains / EC_{50} StrS strains

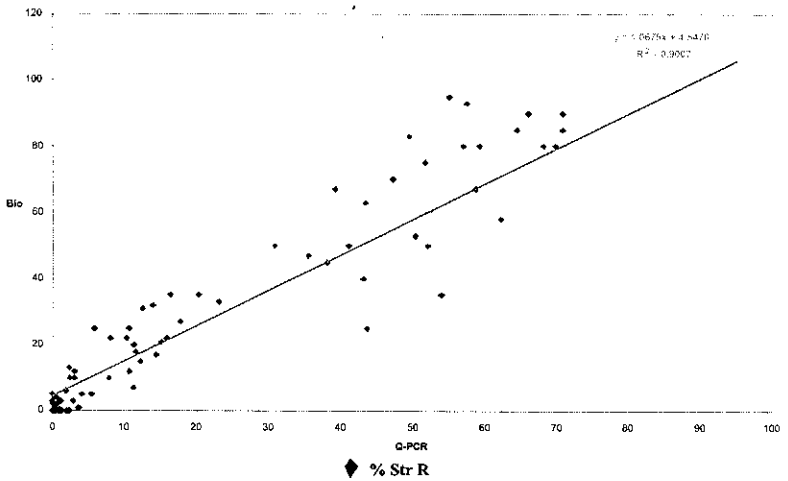

Fig. 1. Comparison of biological (Bio) and molecular (Q-PCR) methods to quantify strobilurin resistance within *Septoria tritici* populations.

We adopted this second approach and our screening was conducted in the presence of azoxystrobin or famoxadone (0.25 or 0.5 mg L^{-1}). A good correlation was obtained when the same field samples were submitted to both molecular and biological tests (Fig. 1).

From our data we were not able to detect QoI-resistant strains in France between 1997 and 2002. However, during 2003, despite unfavourable climatic conditions, a major shift in the French populations of *S. tritici* was recorded. Indeed, QoI resistant strains were detected in more than 50% of the tested samples. Since 2003, this shift has become more pronounced with, 66% of the tested samples exhibiting more than 50% of StrR (Table 1) in 2005.

In terms of selection pressure, it appeared that in most trials, strobilurins alone or in mixture selected QoI resistant strains. However, when azoxystrobin alone at 250 g ha^{-1} was compared to the mixture azoxystrobin at 125 g ha^{-1} and epoxiconazole 62.5 g ha^{-1}, the selection pressure was

lower in the latter (Table 3). In contrast, DMIs and chlorothalonil alone appeared neutral towards QoI resistance (data not shown). In the small trial plots (20–50 m^2) used in these studios suggest that pycnidiospores probably play a major role in the vertical progression of *S. tritici* (Leroux *et al.*, 2005 *a,b*).

Table 3. *Selection pressure exerted by a strobilurin alone or in mixture with a triazole towards QoI resistant strains*

Localization of trials[a] Department n°	Percentage of Str R strains		
	Control	azoxystrobin 250 g.ha-1	azoxystrobin + epoxiconazole 125 + 62.5 $g.ha^{-1}$
35	1	66	48
53	5	60	25
59	5	85	25
22	6	60	17
91	13	52	18
18	25	90	68
27	26	85	50

These trials were conducted in 2004 within a network coordinated by Arvalis. Fungicides were applied once and each condition was replicated 3 or 4 times; the surface of each plot was between 20 and 50 m^2. Resistance analysis was realised from a bulk of these replicates.

Table 4 : *Evolution of QoI resistance in a long term trial at INRA - Versaillesa*

Years	Percentage of StrR strains			
	Intensive Production		Organic Farming	
	April-May	June	April-May	June
1998-2002	ND	ND	ND	ND
2003	3	55	ND	0.5
2004	32	85	15	30
2005	50	95	30	45
2006	72	80	50	60

This long-term trial included 4 cropping systems and among them, wheat produced in "Intensive production" was treated by DMIs and strobilurins, whereas no fungicide was applied on "Organic farming" wheat. Each condition was replicated twice and the surface

Regarding a long-term trial established by INRA at Versailles and in long term trails comparing several cropping systems, it has been shown that QoI-resistant strains could invade the "Organic farming" plots (Table 4). In fact, this trial includes the following conditions: -Intensive production -Integrated Pest Management -Permanent covering without tillage -Organic farming, and each plot covered 5000 m^2. These results suggest that the spread of QoI resistance from treated to untreated plots, probably involves ascospores and -that in *S. tritici*, QoI resistant strains are fit.

Resistance to DMIs

The situation with DMIs is totally different from that recorded with benzimidazoles and QoIs. Indeed, we detected several phenotypes exhibiting low to moderate resistance levels; moreover, cross-resistance did not always concern all DMIs. We identified seven phenotypes resistant to DMIs (TriR) distributed into main categories: TriLR (low RLs) and TriMR (medium RLs). Within TriLR strains, TriR1 and TriR3 remain sensitive to triflumizole and fluquinconazole but were

among the most resistant ones towards triadimenol. Among the TriLR strains, TriR2 and TriR4 were resistant to all DMIs whereas in TriR5, cross-resistance did not occur to tebuconazole. The TriMR strains were less susceptible to most DMIs than TriLR strains; the highest difference

Table 5. In-vitro *effects of DMIs towards field strains sensitive (TriS) or resistant (TriR) to these fungicides*

Fungicides	EC_{50} mg.l^{-1} for wild type strains (TriS)[a]	Resistance levels in TriR strains[b]						
		Tri LR					Tri MR	
		Tri R1	Tri R2	Tri R3	Tri R4	Tri R5	Tri R6	Tri R7
Pyrifenox	0.001	3	8	10	27	28	36	37
Triflumizole	0.004	0.3	10	0.8	28	31	333	500
Prochloraz	0.002	3	4	9	7	15	7	1.0
Triadimenol	0.6	17	2	25	7	3	27	24
Tebuconazole	0.01	4	8	14	21	1.8	75	83
Fluquinconazole	0.003	0.5	3	1.3	6	14	20	19
Flusilazole	0.006	4	5	21	13	19	32	42
Metconazole	0.002	4	3	9	10	8	16	15
Epoxiconazole	0.002	4	3	14	6	9	26	19
Prothioconazole	0.04	-	3	4	4	5	8	7

a EC_{50} values towards germ tube elongation (see Material and Methods)
b Resistance levels or RLs : EC_{50} TriR strains / EC_{50} TriS strains

Table 6. *Amino-acid polymorphism in the CYP51 gene sequence (between codons 340 and 513) from field strains of Septoria tritici sensitive (TriS) or resistant (TriR) to DMIsa*

Phenotypes[b]	Positions of codons				
	381	459	460	461	513
TriS	I	Y	G	Y	N
TriR2	I	D	G	Y	N
TriR3	I	Y	G	Y	N or D
TriR4	I	-	-	Y	K or E
TriR5a	I	Y	G	S	N
TriR5b	I	-	-	Y	K
TriR6a	V	S, D or N	G	Y	N
TriR6b	V	Y	H	Y	N or D
TriR7	V	-	-	Y	K or E

a The putative protein encoding CYP51 gene from wild type strains comprises 544 proteins (Cools *et al.*, 2005*a,b*)
b Among DMI-resistant strains (TriR), TriR1, TriR2, TriR3, TriR4 and TriR5 exhibited low resistance levels (TriLR) whereas TriR6 and TriR7 are moderately resistant (TriMR) (see Table 5)

being recorded with triflumizole. However, prochloraz did not behave like other DMIs as RL values remained low in TriMR strains. In addition within these strains, some were weakily resistant (TriR6) whereas others remained sensitive to this imidazole derivative (TriR7) (Table 5).

Amplifications of *S. tritici* genomic DNA with primers A and B yielded in a single major product of PCR of about 0.6 kb size. This fragment potentially encodes a 202 amino-acid polypeptide between codon 317 and 518 of CYP51. The remaining DMI-resistant strains could be divided

Table 7. *Selection pressure exerted by a triazole alone or in mixture with a strobilurin towards resistance to QoIs and DMIsa*

Localisation of trials Department n°	Treatments	% StrR strains	% TriMR strains[b]	% TriR phenotypes[b]			
				TriR4	TriR5	TriR6	TriR7
57	Control	27	20	70	10	20	ND
	Epoxiconazole	32	75	25	ND	55	20
	Epoxiconazole + pyraclostrobin	95	70	25	5	60	10
89	Control	45	50	50	ND[c]	40	10
	Epoxiconazole	55	80	5	15	70	10
	Epoxiconazole + pyraclostrobin	95	75	25	ND	65	10
22	Control	40	40	60	ND	30	10
	Epoxiconazole	35	90	10	ND	75	15
	Epoxiconazole + pyraclostrobin	90	85	15	ND	50	35
88	Control	15	15	85	ND	10	5
	Epoxiconazole	ND	90	10	ND	80	10
	Epoxiconazole + pyraclostrobin	75	85	15	ND	45	40

a These trials were conducted in 2005, within a network coordinated by Arvalis. Fungicides were applied twice (epoxiconazole 75 g ha^{-1} or epoxiconazole + pyraclostrobin 50 + 50 g ha^{-1}) and each condition was replicated 3 or 4 times. Resistance analysis was realised from a bulk of these replicates.
b The TriR phenotypes are described in Table 5.
c ND : Not Detected

two groups according to the presence or the absence of a double deletion at positions 459 and 460 (ΔY459/G460).

Where isolates had a complete sequence, it appeared that all of them carried a mutation at positions 459, 460 or 461. The only tested TriR2 strain showed aspartate (D) instead of glycine (G) at position 460. Within TriR5 strains, some of them (TriR5a) had serine (S) instead of tyrosine (Y) at position 461. Within the TriR6 strains, the only modification at position 461 was the presence of a histidine (H) instead of a tyrosine (Y), whereas at position 459, a tyrosine (Y) was replaced by either aspartate (D), asparagine (N) or serine (S). In addition, all the TriR6 strains had valine (V) instead of isoleucine at position 381 (Table 6). All strains having the double deletion ΔY459/G460 were resistant to DMIs and were allocated within TriR4, TriR5 (TriR5b) and TriR7 phenotypes. Moreover, all of them had a lysine (K) or a glutamate (E) at position 513 instead of an asparagine (N) or an aspartate (D) in the strains without this deletion. No specific differences were observed between TriR4 and TriR5b strains whereas TriR7 ones were characterized by the presence of a valine (V) instead of an isoleucine (I) in position 381 (Table 6).

According to the relationship between genotypes and phenotypes, it appeared that modifications at positions 459, 460 and/or 461 determined low RLs, whereas the change from isoleucine (I) to valine (V) at position 381 was found to be essential in TriMR strains. By analogy to work

Table 8. *Selection pressure exerted by a triazole alone or in mixture with prochloraz or a strobiluren towards resistance to QoIs and DMIsa*

Localization of trials Department n°	Treatments	% StrR strains	% TriMR strains[b]	% TriR phenotypes[b]			
				TriR4	TriR5	TriR6	TriR7
32	Control	2	75	12	13	35	40
	Epoxiconazole	2	95	5	ND[c]	25	70
	Epoxiconazole + prochloraz	ND	30	40	30	ND	30
	Epoxiconazole + pyraclostrobin	35	90	10	ND	20	70
17	Control	20	40	20	40	25	15
	Epoxiconazole	5	87	ND	13	12	75
	Epoxiconazole + prochloraz	ND	ND	20	80	ND	ND
	Epoxiconazole + pyraclostrobin	30	80	5	15	5	75
28	Control	95	70	ND	30	60	10
	Epoxiconazole	95	60	ND	40	60	ND
	Epoxiconazole + prochloraz	100	15	20	65	15	ND
	Epoxiconazole + pyraclostrobin	100	80	ND	20	60	20
95	Control	100	80	ND	20	80	ND
	Epoxiconazole	90	80	ND	20	80	ND
	Epoxiconazole + prochloraz	100	45	ND 100	55	45	ND
	Epoxiconazole + pyraclostrobin	95	100	ND	ND	100	ND

a These trials were conducted in 2006 within a network coordinated by Arvalis. Fungicide were applied twice (epoxiconazole 75 g ha^{-1} ; epoxiconazole + prochloraz 50 + 315 g ha^{-1} or epoxiconazole + pyraclostrobin 50 + 50 g ha^{-1}) and each condition was replicated 3 or 4 times. Resistance analysis was realized from a bulk of these replicates.

b The TriR phenotypes are described in Table 5.

c ND : Not Detected

valine (V) was always associated with alteration at codons 459, 460 or 461 suggests that alone it has negative effects on the sterol 14α-demethylase function. In addition, according to the response of TriMR strains towards prochloraz (especially TriR7), the exchange of isoleucine (I) to valine (V)seems to increase specifically the binding capacity of this imidazole derivative. Regarding the strains bearing the ΔY459/G460 deletion, it is still not clear if they belong to an independent genetic entity, naturally resistant to DMIs.

To determine the distribution of DMI-resistance strains of *S. tritici* in France, field populations were screened in the presence of pyrifenox (0.01 mg L^{-1}), triflumizole (0.003, 0.3, 1 mg L^{-1}),

conducted by Poudust *et al.* (2001) on crystallised *Mycobacterium tuberculosis* Cyp51p, this isoleucine constitutes part of a putative sterol / DMI binding site. The fact that the change for a prochloraz (0.003 mg L^{-1}) and tebuconazole (0.05 mg L^{-1}). With this approach, we were able to characterize the four predominant phenotypes: TriR4, TriR5 (TriLR) and TriR6, TriR7 (TriMR). The samples were also tested with epoxiconazole at 0.2 mg L^{-1}, in order to detect strains highly resistant to DMIs. Up to now, we have never detected such a phenotype in France. The survey conducted in France since 1997 indicated that in *S. tritici*, DMI-resistance is generalised (Table 1). We do not know exactly at what time the shift of French populations took place, but according to other data recorded in Europe, this phenomenon seemed to occur in the early 1990s. Within DMI-resistant strains, according to our monitoring conducted in France over the last two years, it appeared that in many locations TriMR strains (and especially TriR6) predominate. The few date available between 1997 and 2000 indicate that most DMI-resistant strains belonged to TriR4 type (TriLR category) (O Leroux, unpublished data). Surprisingly in *S. tritici*, the shift from TriLR to TriMR strains coincides with the emergence of resistance to QoIs (Gisi *et al.*, 2005).

In terms of the practical impact of DMI-resistance, the situation is less critical than for QoIs. Indeed, in spite of the widespread distribution of DMI-resistance, the efficacy of triazoles and especially epoxiconazole remained high correct. For instance, the results, obtained within a network of trials conducted by AFPP (French Association for Plant Protection), showed that the efficacy of epoxiconazole (two treatments by 125 g ha^{-1}) ranged from 76 to 87% over the period 1997–2005 without any significant decline. At the same time, azoxystrobine (two treatments by 250 g ha^{-1}) was equivalent to epoxiconazole between 1997 and 2003, but its average efficacy dropped to 50% in 2004 and 20% in 2005 because of QoI resistance (unpublished data; Leroux *et al.*, 2005*a*).

Regarding selection pressure, the data collected in trials conducted in 2005 and 2006 revealed that the treatment with a triazole like epoxiconazole selects TriMR strains (Table 7). Moreover, in several locations, programs including prochloraz induced decreases in the frequency of TriMR strains (Table 8). This result could be explained by the fact that TriMR strains are equally or more sensitive to prochloraz than TriLR ones.

Conclusion

In *S. tritici*, as in many other fungi, resistance to benzimidazoles and QoIs (strobilurins) is typically "discrete" or "qualitative". In both cases, it is determined by a point mutation in the gene encoding β-tubulin and cytochrome b, the respective target sites of benzimidazoles and QoIs. These monogenic and monoallelic resistances determine high resistance levels to all members of these two classes of fungicides and lead to failures in the practical control of *S. tritici*. Regarding DMIs, resistance seems to be "quantitative". A polygenic process often determines such a "mulitstep" pattern. However, in *S. tritici*, this phenomenon seems to be monogenic and it combines multi and polyallelic resistance; mutations concern CYP51, the gene encoding the target site of DMIs. Cross-resistance was not always observed between all DMIs; this was particularly the case for prochloraz, triflumizole, fluquinconazole and tebuconazole. Under field conditions, the efficacy of most DMIs remains good, even in locations where resistance is generalised. However, a survey of *S. tritici* populations must be done in the future in order to track strains highly resistant to DMIs.

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