Recent evolution of fungicide resistance in French populations of Mycosphaerella graminicola

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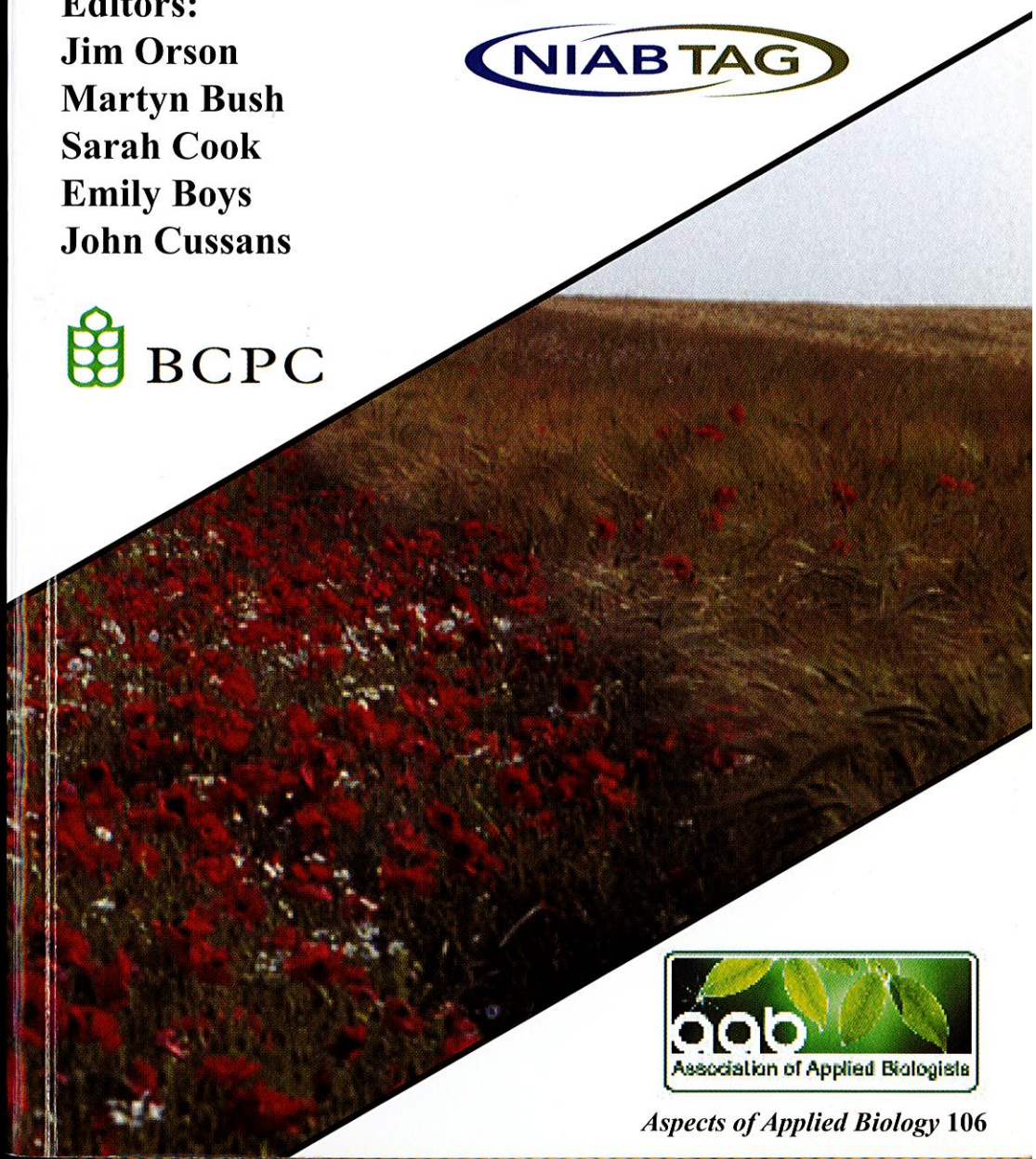

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Summary

Sterol 14α-demethylation inhibitors (DMIs) have been widely used in many European countries and erosion of efficacy, correlated with significant shifts in sensitivity of *M. graminicola* populations, has been recorded for most of them. Recently, strains highly resistant to DMIs have been isolated from French but also other European populations. This work aims to characterize these *M. graminicola* field isolates and to evaluate the risk of practical resistance if they were to progress in populations. Target alteration, linked to one or several changes in the gene CYP51, encoding sterol 14α-demethylase, was the basic resistance mechanism in all DMI-resistant strains. Changes in CYP51, combined with the overexpression of drug efflux transporters probably result in multidrug resistance in the more resistant phenotypes. Moreover, some isolates moderately or highly resistant to DMIs harbour an insertion in the CYP51 promoter and/or new combinations of already known mutations in the target gene. An inoculated field trial showed that efficacy of all fungicides may be partially reduced when these strains are dominant.

Key words: *Mycosphaerella graminicola*, azole, acquired resistance, CYP51, multidrug resistance, resistance dynamics, efficacy

Introduction

Septoria leaf blotch, caused by *Mycosphaerella graminicola* (anamorph *Septoria tritici*) is the major foliar disease of winter wheat in France and Western Europe. Sterol 14α-demethylation inhibitors (DMIs) have been the key components of fungicide strategies used to control this disease in the last 25 years. Most are triazole derivatives (e.g. cyproconazole, epoxiconazole, fluquinconazole, flusilazole, metconazole, tebuconazole), but this class of fungicides also includes prochloraz (imidazole) and prothioconazole (triazolinethione).

Sterol 14α-demethylase (CYP51) is a cytochrome P450 required for sterol biosynthesis in various phyla, including fungi. It is the biochemical target of DMIs, which are thought to inhibit cytochrome P450 by binding to the active site "cysteine pocket" via a protonated nitrogen atom coordinated with the haem iron.

In pathogens of plants and humans, DMI resistance may be determined by (1) alterations in CYP51, decreasing the affinity of DMIs for their target site (2) CYP51 overexpression, resulting in high levels of sterol 14α-demethylase and (3) an increase in the efflux of DMIs, due to the upregulation of ABC (ATP-binding cassette) or MFS (major facilitator superfamily) transporters in the membrane. Most of these efflux pumps can transport various unrelated compounds and their overproduction

may lead to multiple drug resistance (MDR). A combination of these mechanisms, leading to the polygenic control of DMI resistance, is commonly found in clinical isolates of *Candida albicans* (Akins, 2005).

In *M. graminicola*, DMI resistance in European countries resulted mostly from changes in CYP51, at least until 2007 (Cools *et al.*, 2005, Leroux *et al.*, 2007). However, a continuous shift in sensitivity to DMIs has been observed recently, consistent with additional mechanisms, and this "quantitative" or "multiple-step" resistance is thus considered to be polygenic (Cools & Fraaije, 2008; Chassot *et al.*, 2008). Eight categories of strains (TriR1-TriR8) displaying reduced sensitivity to DMIs have been characterised in previous studies and were classified into two main groups, TriLR (TriR1-TriR5) and TriMR (TriR6-TriR8) (Leroux *et al.*, 2007; Fig. 2). This classification is based on *in vitro* responses to various families of DMIs including pyridines (e.g. pyrifenox), imidazoles (e.g. prochloraz, triflumizole) and triazoles (e.g. difenoconazole, epoxiconazole, fluquinconazole, propiconazole, tebuconazole, triadimenol), and changes in the target encoded by CYP51. Monitoring in 2008, 2009 and 2010 resulted in the identification of new strains, more resistant to DMIs than those found before 2010. The aim of this study was to characterize these new isolates, for their phenotypic and genotypic characteristics but also also to evaluate their progression in populations and the associated risk of practical resistance.

Material and Methods

Origin of samples

Nineteen isolates of *Mycosphaerella graminicola* were collected in 2009 in France and UK after isolation from diseased wheat leaves. Irish isolates are a kind gift from Professor O'Sullivan (Teagasc). Isolates collected before 2007 were used as reference isolates representing the various phenotypes TriR1-TriR8 (Leroux *et al.*, 2007). All isolates were kept as mono-conidial cultures on a medium containing 20 g L^{-1} malt, 5 g L^{-1} yeast extract and 12.5 g L^{-1} agar, at 17°C in the dark.

Bulk populations were produced from at least 20 diseased leaves, collected in field trials all over France. A few samples were also collected in the UK. These samples enabled assessing the frequency of the various phenotypes in populations.

Resistance phenotype characterization

Sensitivities of the single conidia isolates towards DMI inhibitors were determined at different concentrations, following a geometric progression of ×2, ×2.5 or ×3, on a medium containing glucose 10 g L^{-1}, K_2HPO_4 2 g L^{-1}, KH_2PO_4 2 g L^{-1} and agar 12.5 g L^{-1}. Germ-tube elongation was assessed microscopically after 48 h incubation at 17°C in the dark. EC_{50} values and resistance factors (RFs) were determined as described previously (Leroux & Walker, 2011).

Frequency of resistance in bulk populations were determined using discriminate doses of fungicides (Leroux *et al.*, 2007), including high doses of epoxiconazole, prothioconazole, prochloraz and pyrifenox that detect only novel TriR strains.

Molecular procedures

DNA from the isolates was extracted using a sarcosyl-based protocol. PCR-amplification was performed for the CYP51 gene as previously described (Leroux *et al.*, 2007). CYP51 promoter insertion was checked using the protocol from Chassot *et al.* (2008) as it was suggested to be linked to CYP51 overexpression, by the author.

Inoculated field trial

A field trial, cultivar Tremie, located in Boigneville (Paris area, France), received a cover spray of chlorothalonil (750 g ha^{-1}) at stage GS31–32. The trial was then inoculated three times with spore suspensions of either TriR6, TriR9 or MDR-6 strains (see below for description), respectively

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Table 1. *Effect of DMIs on germ-tube elongation of* Mycosphaerella graminicola *field isolates*[a]

Fungicide	EC_{50} (mg L^{-1}) *Tris*	Resistance factors (RFs)[b]											
		Tri R4	*Tri R5*	*Tri R5+*	*Tri R6*	*Tri R7*	*Tri R8*	*Tri R8+*	*Tri R9*	*Tri R11a*	*MDR-6*	*MDR-7*	*MDR-10*
Pyrifenox	0.0013	23.1	27.7	126.9	36.2	30.8	40.0	123.1	192.3	153.8	192.3	246.2	230.8
Fenarimol	0.035	5.7	5.7	6.3	17.1	17.1	22.9	22.9	7.6	14.3	114.3	114.3	28.6
Prochloraz	0.0018	6.7	15.0	27.8	6.7	1.5	0.8	2.3	66.7	22.2	111.1	122.2	166.7
Triflumizole	0.0036	27.8	30.6	59.7	333.3	194.4	722.2	111.1	68.8	833.3	13889	1111	416.7
Bromuconazole	0.017	14.7	23.5	33.8	47.1	41.2	47.1	176.5	79.4	117.6	253.3	264.7	88.2
Cyproconazole	0.049	4.3	8.5	18.3	11.2	7.6	13.1	40.8	16.3	34.7	112.2	61.2	40.8
Difenoconazole	0.00025	20.0	1.6	2.8	32.0	32.0	60.0	60.0	6.3	6.0	800.0	600.0	28.0
Epoxiconazole	0.0020	5.0	8.5	18.3	25.5	11.0	23.0	75.0	29.9	60.0	225.0	210.0	60.0
Fenbuconazole	0.0020	12.5	1.5	5.9	50.0	50.0	75.5	175.0	18.6	15.0	650.0	500.0	40.0
Fluquinconazole	0.0031	6.5	14.2	34.7	20.3	14.5	22.6	64.5	75.8	103.2	387.1	387.1	112.9
Flusilazole	0.0057	12.3	19.3	21.9	31.6	40.4	43.9	140.4	61.4	105.3	438.6	350.9	122.8
Hexaconazole	0.0045	5.6	4.4	5.3	8.9	6.7	8.9	17.8	8.9	11.1	55.6	44.4	26.7
Metconazole	0.0020	10.0	8.0	7.9	15.5	10.0	17.5	50.0	23.6	30.0	110.0	150.0	40.0
Propiconazole	0.0037	12.2	20.5	29.1	35.1	27.0	54.1	94.8	70.9	54.1	459.5	270.3	162.2
Tebuconazole	0.011	18.2	1.8	6.4	74.5	51.8	90.9	363.6	12.0	18.2	636.4	500.0	45.5
Triadimenol	0.57	7.4	3.3	6.6	27.2	21.1	26.3	>43.9	9.6	26.3	>43.9	>43.9	26.3
Prothioconazole	0.040	3.5	5.3	10.2	7.8	7.0	7.3	12.5	16.3	25.0	25.0	25.0	12.5

[a]Results for TriR1-R3 strains were not included because these phenotypes were not detected any more during the last 3 years.

at stage GS39, GS45 and GS51. The second fungicide treatment was applied at stage GS45. The disease progression was noticed weekly, as was the proportion of leaf surface covered by septoria necrosis, in the non inoculated control plot, in the inoculated and treated plots.

Results and Discussion

Characterization of novel TriR strains

Thorough examination of isolated novel TriR strains revealed various new phenotypes (Leroux *et al.*, 2011), different to those previously described (Leroux *et al.*, 2007) (Table 1).

Among them, phenotypes TriR5+, TriR8+, TriR9 and TriR11a exhibited very high resistance factor (> 100) towards one or a few DMIs, especially pyrifenox. TriR5+ and TriR8+ exhibit the same cross-resistance pattern that TriR5 (no or weak cross-resistance with tebuconazole) and TriR8 (no or weak cross-resistance with prochloraz), respectively, but with either RFs (Table 1). Indeed, these isolates share the same changes in CYP51 as the phenotypes they are derived from (Fig. 1). No CYP51 promoter insertion was observed within these strains. Their additional resistance mechanism is still unkown. Alternatively, TriR9 isolates were highly resistant to pyrifenox and newly combined the V/C136A and S524T changes in CYP51. This phenotype was most commonly found in Ireland and South-West England in 2009, in addition to France. TriR11a isolates exhibited very high resistance towards pyrifenox, bromuconazole, fluquinconazole and flusilazole (Table 1) and exhibited the new combination of changes V/C136A + I381V + D134G in CYP51 (Fig. 2). In addition, CYP51 promoter insertion was found in some TriR11a isolates.

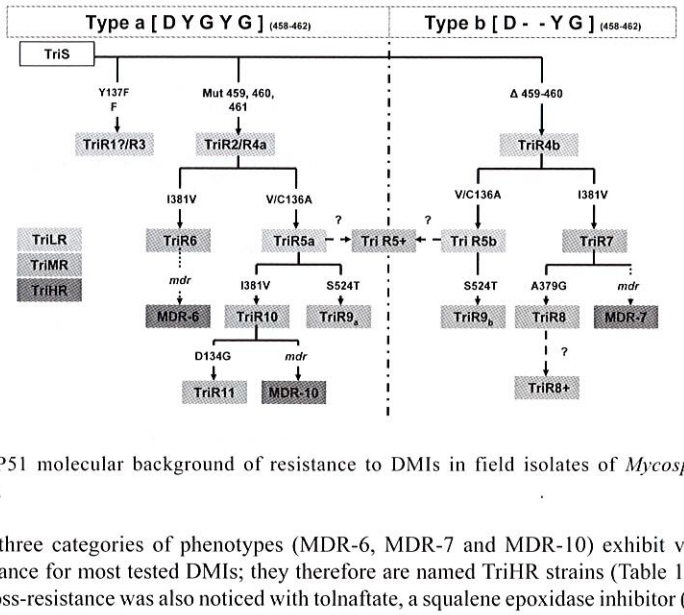


Fig. 1. CYP51 molecular background of resistance to DMIs in field isolates of *Mycosphaerella graminicola.*

The last three categories of phenotypes (MDR-6, MDR-7 and MDR-10) exhibit very high cross-resistance for most tested DMIs; they therefore are named TriHR strains (Table 1; Fig. 1). Positive cross-resistance was also noticed with tolnaftate, a squalene epoxidase inhibitor (RF=25), strobilurins (RF=4-10, when compared to QoIR isolates), inhibitors of succinate deshydrogenase (SDHIs) (RF=5-15) but not with chlorothalonil, fenpropimorph, fludioxonil, cyprodinil and fenhexamid (Leroux & Walker, 2011). Examination of CYP51 sequence did not reveal any new mutation or combination of mutations in these strains, and confirmed their resistance mechanism had been selected in CYP51 TriR6, TriR7 or TriR10 genetic background, respectively for MDR-6,

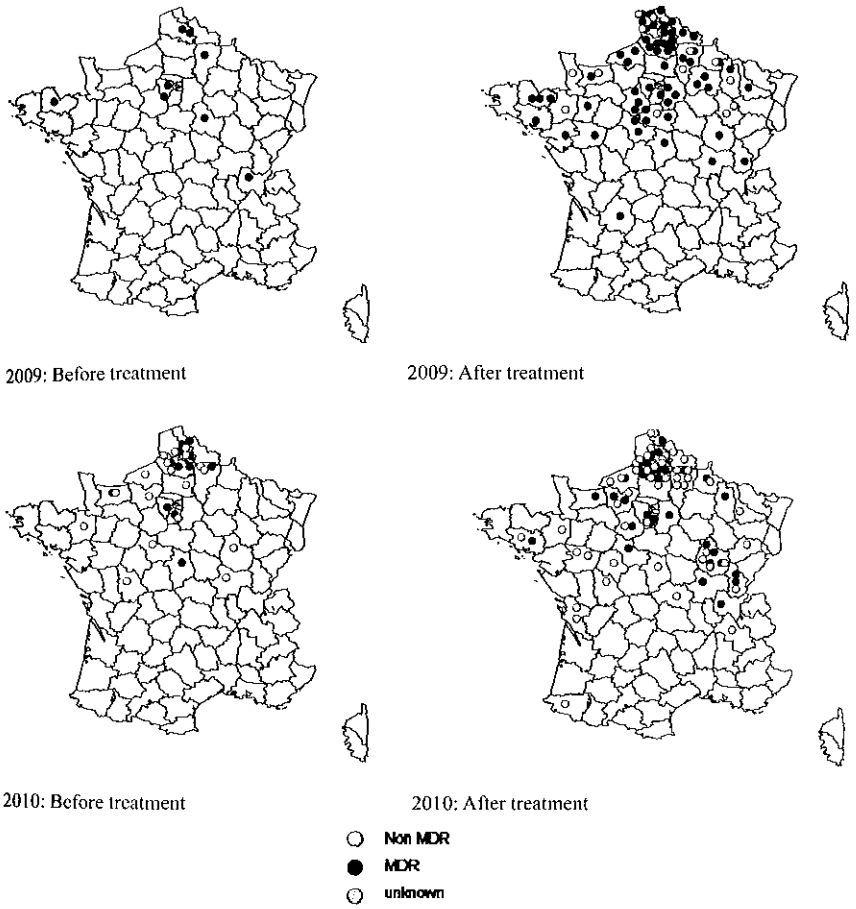


Fig. 2. Occurrence of field trials with the presence of new TriR strains in populations in French populations of *S. tritici* in 2009 and 2010.

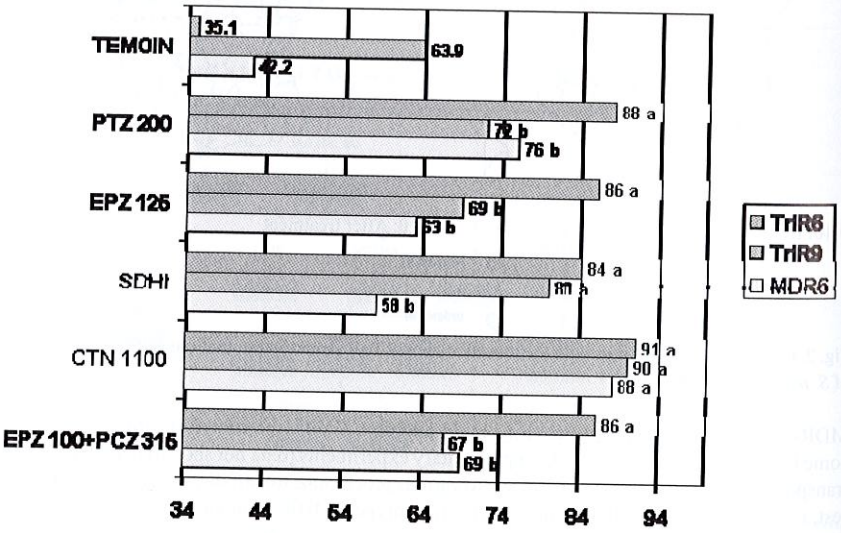
MDR-7 and MDR-10 phenotypes (Fig. 1). In addition, CYP51 promoter insertion was found in some but not all of these isolates. Complementary experiments (data not shown) revealed that drug transporter modulators reduced resistance to various prochloraz, tolnaftate or boscalid in our in vitro test, for strains from the MDR-6 and MDR-7 phenotypes (MDR-10 not tested). All together, these data suggest that multiple resistance related to overexpression of one or several MFS- or ABC-transporters occurring in these isolates. Moreover, multidrug resistance was already suggested in the laboratory (Roohparvar *et al.*, 2008) or field isolate of *M. graminicola*, but without correlation with phenotype (Cools *et al.*, 2005*b*). This would represent the second case of multidrug resistance in phytopathogenic fungi after *Botrytis cinerea* (Kretschmer *et al.*, 2009), whereas, this mechanism is well established for human pathogens (Akins, 2005).

Progression of novel TriR strains in French and other European populations

Novel TriR strains were first observed in 2008 in 3% of the bulk populations analysed in our routine monitoring as spores exhibiting long germ tubes at high doses of various DMIs. These isolates

were successfully isolated in 2009. At that time, they were present in 13% of the tested populations, with a mean frequency of 0.6% in the whole sampling and 4.9% in positive plots. The maximum observed frequency was 40% from a positive location in Brittany. These emerging phenotypes were observed again in 2010 at higher frequencies. In 2010, they were detected in 30.3% of the tested samples (20.4% for non MDR strains, i.e. TriR5+, TriR8+, TriR9 and TriR11, and 11.7% for MDR strains, i.e. MDR-6, MDR-7 and MDR-10). Their mean frequency was 4.5% in the whole sampling (3.2% for non MDR and 1.3% for MDR) and 14.7% in positive plots (15.8% for non MDR and 9.8% for MDR). At last, very high frequencies were observed in a few locations (96% for non MDR and 90% for MDR). Moreover, a few populations collected in the UK in 2009 and 2010 revealed that some of these emerging phenotypes (MDR and non MDR) were also present in this country at low frequencies. More generally, non MDR strains were detected in many European countries at increasing frequencies and many new genotypes (novel combinations of CYP51 mutations) were characterized, after sequencing (G Stammler, BASF, personal communication). Since the molecular mechanism of MDR strains is still unknown, this category of strain can only be detected iby monitoring using phenotyping methods.

As observed in Fig. 2, these emerging phenotypes had a greater occurrence in July 2009 and 2010, i.e. after a mean application of two treatments. As DMIs are the basis of septoria leaf blotch chemical control, this would suggest that these fungicides strongly select these new isolates. As positive cross-resistance is observed in MDR strains between azoles, QoIs and SDHIs. It remains conjecture as to what extent QoIs and SDHIs modes of action have selected for MDR strains.



See Table 1 for the characterization of strains. Frequency of these strains in inoculated control plots were checked at 95, 98 and 95% frequency, respectively.
TEMOIN: disease level in the untreated control
PTZ: prothioconazole
EPZ: epoxiconazole
SDHI: succinate dehydrogenase inhibitor
CTN: chlorothalonil
PCZ: prochloraz

Fig 3. Field efficacy of various fungicides in plots inoculated with strains of *S. tritici* resistant to azoles.

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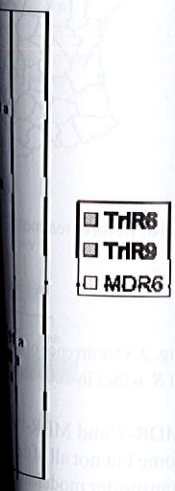

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resistant to azoles.

Efficacy of fungicides in 2010

The generalisation of TriMR strains, at the beginning of 2000, had lead to the erosion of azoles efficiency, which meant they had to be used in mixture. Despite the emergence, in 2008, of new phenotypes highly resistant to azoles, efficacy of programs using the best fungicides remained similar to the previous years standards, although 2010 was a year of low disease pressure (Maumene *et al.*, 2010). These good results seem to contradict the fact that some highly resistant strains are present in populations. Obviously, their still relative scarceness may explain this observation. It is difficult to predict if these strains will progress in populations up to level at which they will affect fungicides efficacy. To answer this question, an inoculated field trial was conducted in Boigneville in 2010. Results are presented in Fig. 3. Analysis of the population structure in inoculated control plots revealed that the inoculated strains were dominant (>95%). Efficacy of most triazoles, epoxiconazole, prochloraz, prothioconazole were reduced in the presence of TriR9, and more particularly, or MDR-6 strains. Efficacy of SDHI was reduced only in the presence of the MDR-6 strain. Interestingly, efficacy of fungicides was only partially, and not fully, reduced. As expected, the efficacy of chlorothalonil, a multisite inhibitor, was not affected.

Conclusion

Finally, *M. graminicola* populations keep evolving and new resistant phenotypes can regularly be described, because DMIs fungicides are not likely to be abandoned in wheat disease management programmes. At least two resistance mechanisms, i.e. target alteration and drug transporters overexpression, seem to be responsible for these increasing RFs observed in resistant isolates. They can also cumulate in a single strain, maybe without any evident fitness penalty.

Some of the new phenotypes (TriR5+, TriR8+, TriR9 and TriR11), classified among the TriMR group, may not exhibit high resistance risk and could be controlled by optimized chemical strategies. The situation may be different for TriHR-MDR strains. Further work is needed to understand more accurately how the MDR mechanism is responsible for azoles and other unrelated modes of action, resistance in *M. graminicola*. Especially drug transporters need to be identified, as well as the genome alterations responsible for their overexpression. Practical concerns to estimate their fitness and of the field efficacy losses that may occur are urgently needed, to be able to recommend effective preventive anti-resistance strategies. As these strains exhibit positive cross-resistance between all DMIs subgroups, SDHIs and QoIs, all three families are commonly used on wheat, qualitative and quantitative selective pressure of the various molecules need to be estimated.

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