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Azole and SDHI Sensitivity Status of *Zymoseptoria tritici* Field Populations Sampled in France, Germany and the UK during 2015

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ABSTRACT

Several *Zymoseptoria tritici* populations were sampled in France, Germany and the UK during early season in 2015. Overall, the two French populations were most sensitive to azole fungicides whereas one population in N-Germany contained a high proportion of highly insensitive strains. Further characterisation of strains from this location showed a high frequency of strains overexpressing both CYP51 [L50S-S188N-I381V-Δ-N513K] and the efflux pump encoding gene *MgMFS1*. Complex CYP51 variants with S524T showed high levels of insensitivity to epoxiconazole. Overexpression of *MgMFS1* also conferred insensitivity to SDHIs in *in vitro* testing. Monitoring revealed that several UK field strains were carrying various SdhC alterations (e.g. C-T79N, C-H152R and C-I161S) with different levels of insensitivity to SDHI fungicides. Further monitoring is required to establish if more Sdh and CYP51 variants are evolving and if accumulation of particular variants can have an impact on disease control.

INTRODUCTION

Azole fungicides have been used for more than three decades to control Septoria leaf blotch (SLB) caused by the fungus *Zymoseptoria tritici.* However, the fungus has adapted over time to resist azoles and studies have shown that combinations of three different resistance mechanisms can be present in field isolates (Cools & Fraaije 2013). The most common mechanisms are CYP51 target alterations with up to 9 different amino acid residues simultaneously affected. More recently, overexpression of *CYP51* due to a 120 bp promoter insert (Cools et *al*. 2012) and/or *MgMfs1*, a Major Facilitator Superfamily transporter (efflux pump) (Roohparvar et *al*. (2007), due to a 519 bp promotor insert have been shown to contribute to azole insensitivity (Omrane et *al*. 2015). Due to resistance to Quinone outside Inhibitors (QoIs) and loss of azole efficacy, Succinate DeHydrogenase Inhibitors (SDHIs) and multi-site inhibitors have become key components in spray programmes to control SLB. SDHIs are at risk of resistance development and continued monitoring of fungicide sensitivity in field populations is required to inform optimal disease management strategies. Here we present the latest genotype-to-phe-

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notype relationships for azole and/or SDHI insensitive field strains of *Zymoseptoria tritici* sampled at 7 different locations in France (2), Germany (2) and the UK (3) during spring 2015 and discuss the practical implications for Septoria leaf blotch control.

MATERIAL AND METHODS

Sampling and fungicide sensitivity testing

Leaves (approx. 100 per location) were sampled from winter wheat fields at seven different locations in N-Europe during January-April at the start of the season in 2015 (Table 1).

Table 1 Sampled *Z. tritici* populations from wheat fields during early spring 2015

Sample code and location	Cultivar	Foliar fungicide treatment	Number of strains
UK-1; Harpenden, Hertfordshire, UK	Dickens	none	53
UK-2; Tilney St. Lawrence, Norfolk, UK	Santiago	None	24
UK-3; Warminster, Wiltshire, UK	Leeds	Two sprays (mixture of azoles and chlorothalonil)	42
FR-1; Chahaignes, LeMans, France	Cellule	None	44
FR-2; Reims, France	Trapez	None	46
GE-1; Veelböken, Mecklenburg, Germany	Tobac	None	44
GE-2; Alfhausen, Osnabruck, Germany	JB Asano	None	43

In total, 296 strains were isolated and tested for fungicide sensitivity (epoxiconazole, tebuconazole, prochloraz and bixafen) using OD measurements in 96-well microtitre plates as described by Fraaije et *al*. (2012). To check for altered efflux pump activity and QoI resistance, growth in the presence of 5 ppm of tolnaphtate and azoxystrobin, respectively, was determined for all isolates from populations UK-1, FR-1, GE-1 and GE-2 and a small selection of isolates from the other populations.

Table 2 PCR primer sequences, amplification targets and reaction conditions

Primer sets and sequences (5'-3')[1]	Target amplification	Size[2] (bp)	PCR kit and annealing temperature
51F1: TTCTCCCGGAACATTGACAT 51R1: TGCATACCCACACCAATTCT	*CYP51*	~1958	Phusion, 60°C
SdhBF: TAAACACTCCACGCCTCACG SdhBR: GTCTTCCGTCGATTTCGAGAC	*SdhB*	1270	Phusion, 63°C
SdhCF: CTACAARAAMGCCAAMCCCAAC SdhCR: ATGTTGGCACAGAAGCTCAC	*SdhC*	~749	Easy-A, 57°C
SdhDF: CGGGAATAACCAACCTCACT SdhDR: CCTCACTCCTCCAAACCGTA	*SdhD*	840	Phusion, 57°C
51PF1: GTGGCGAGGGCTTGACTAC 51PR1: CGCGAGGACTTCCTGGA	*CYP51* promoter insert	≥435, variable	Red Hot, 55°C
MFF: AAGGTAGGTGAACACCTTATACTC MFR: TTCTTGCTGAAGAAGCGCATGGTTGT	*MgMFS1* promoter insert	≥490, variable	Red Hot, 62°C

[1]Primer SdhBF designed by Dubos et *al.* (2012), primers SdhDF and SdhDR developed by Dooley et *al.* (2016) and primers 51PF1 and 51PR1 reported by Cools et *al*. (2012); [2]For the *CYP51* and *MgMFS1* promoter insert PCR reactions, the amplicon size without insert is shown. Both reactions can result in differences sizes due to isolate-dependent insert length differences for both reactions.

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Genotyping assays

DNA extractions and PCR reactions were carried out with Red Hot *Taq* DNA Polymerase (Thermo Scientific), Phusion High Fidelity Polymerase (Finnzymes Oy) or Easy-A high fidelity PCR cloning enzyme (Agilent) kits and cycling programmes as described previously using primer sets and the PCR conditions listed in Table 2. PCR products were sequenced using the PCR amplification primers; except for *CYP51* where a third primer, 51S1 (5'-AGAAGTTCGCATCGAC-3'), was also used in addition to the two PCR primers to cover the whole area of the genes were key mutations have been reported.

RESULTS AND DISCUSSION

Fungicide sensitivity testing

The fungicide sensitivity tests showed that the two French populations were the most sensitive populations to all three azoles tested when compared to the other populations, with most strains showing EC_{50}s below 1.0; 0.2 and 5.0 ppm for epoxiconazole, prochloraz and tebuconazole, respectively (Fig. 1). German population GE-2 was much more sensitive than GE-1. Amongst all populations tested, GE-1 was the most insensitive population to both prochloraz and tebuconazole, with the highest number of strains showing EC_{50} values greater than 0.5 and 5.0 ppm, respectively. UK-1 was the most azole sensitive UK population tested, followed by UK-2 and UK-3. UK-3 was the most epoxiconazole insensitive population of all populations tested, with the majority of strains showing EC_{50} values greater than 1.0 ppm. However, this population was already exposed twice to sprays with azole mixtures (Table 1) which most likely shifted the populations to less sensitive due to selection of particular genotypes. With regard to bixafen sensitivity, population GE-1 was clearly the most insensitive population with 30 % of the strains tested showing EC_{50} values greater than 0.5 ppm (Fig. 1). QoI resistant strains were not detected in populations UK-1 and FR-2, but 2, 11 and 16 % of the strains from populations GE-1, GE-2 and FR-1 were able to grow in the presence of 5 ppm azoxystrobin. The frequency of strains able to grow in the presence of 5 ppm tolnaphtate was 0, 2, 2, 9 and 34 % for populations FR-1, FR-2, UK-1, GE-2 and GE-1 respectively, but not all strains were growing equally fast, indicating perhaps different expression levels of *MgMFS1* or a phenotype caused by alteration in activity of other efflux pumps. There was a clear correlation between tolnaphtate and bixafen sensitivity, with 18 strains out of 20 with an EC50 $\geq$ 0.5 ppm able to grow well in the presence of 5 ppm tolnaphtate.

Genotyping

The *CYP51* gene was amplified and sequenced from approximately 30 randomly selected isolates of populations UK-1 (30), FR-1 (28) and GE-1 (30). Table 3 shows the two most frequently occurring CYP51 variants in these populations, together with their sensitivity ranges and corresponding resistance factor for the three different azoles tested. CYP51 variants [L50S- I381V-Y461H], [L50S-S188N-I381V-Δ-N513K] and [L50S-D134G-V136A-I381V-Y461H] were detected in, respectively, 7, 1 and 9 strains of population FR-1. Variant [L50S- I381V-Y461H] was not detected in the tested UK-1 and GE-1 strains. Eleven and 12

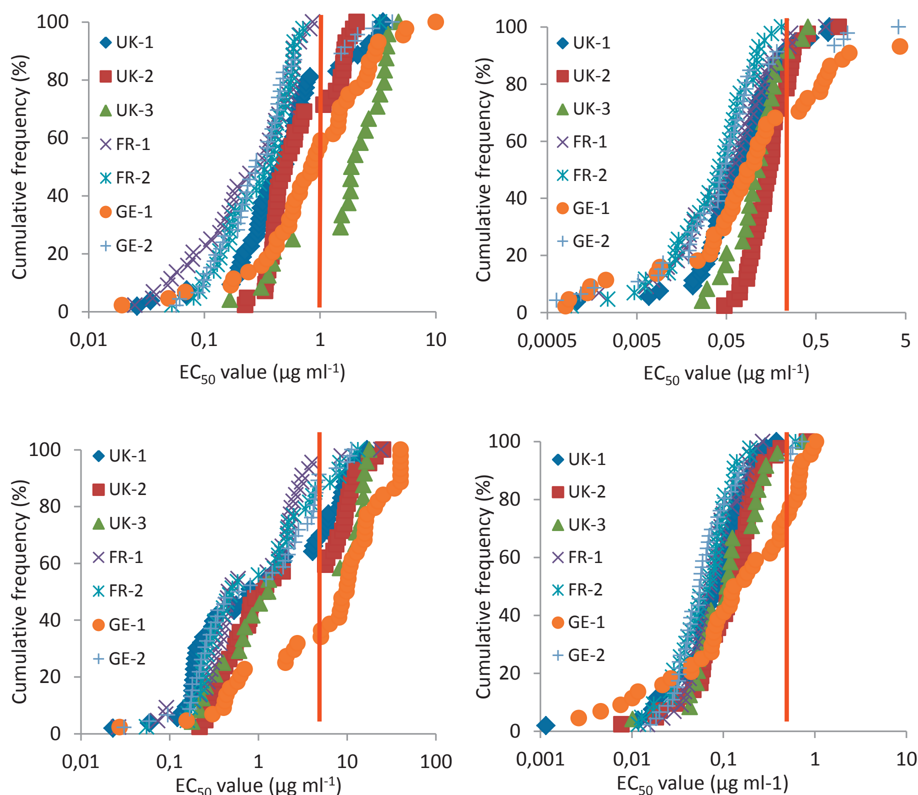

Figure 1 Sensitivity of *Z. tritici* strains to epoxiconazole (A), prochloraz (B), tebuconazole (C) and bixafen (D). Isolates are ranked according to increasing EC_{50} values (cumulative). Codes represent 7 field populations sampled at different locations (see Table 1)

strains of population UK-1 carried variant [L50S-D134G-V136A-I381V-Y461H] and [L50S-S188N-I381V-Δ-N513K], respectively. [L50S-D134G-V136A-I381V-Y461H] and [L50S-S188N-I381V-Δ-N513K] were detected in, respectively, 7 and 14 strains of population GE-1. The 120 bp *CYP51* promoter insert was detected in 12 strains with [L50S-S188N-I381V-Δ-N513K] and two of these strains also overexpressed MgMFS1 due to a 519 bp indert of the *MgMFS1* promoter. The presence of a 519 bp *MgMFS1* promoter insert was also confirmed in one strain with variant [L50S-D134G-V136A-I381V-Y461H]. Isolates with overexpression of *MgMFS1* are less sensitive to azoles but not to the SDHI bixafen. *MgMFS1* overexpressing isolates are less sensitive to both azoles and bixafen. Azole sensitivity is most reduced when isolates are able to overexpress both *CYP51* and *MgMFS1*.

A few highly epoxiconazole and/or bixafen insensitive strains were also further characterised from other populations. Strains carrying complex CYP51 variants with S524T such as [L50S-V136A-I381V-Y461H-S524T], [L50S-V136C-S188N-A379G-I381V-Δ-S524T] and [L50S-D134G-V136A-A379G-I381V-Δ-N513K-S524T] showed high EC_{50} values for epoxiconazole

Table 3 Most common *Z. tritici* CYP51 variants present in populations UK-1, FR-1 and GE-1. The EC_{50} values ± se are presented together with the resistance factor (RF) in bold. RFs were measured by comparing the sensitivities with four sensitive reference strains, known to have no target or non-target fungicide resistance mechanisms

Azole resistant genotypes	**N[a]**	**Epoxiconazole sensitivity**	**Prochloraz senstivity**	**Tebuconazole sensitivity**	**Bixafen sensitivity**
L50S-I381V-Y461H	7	0.166±0.017 **58**	0.031±0.004 **2**	0.377±0.022 **5**	0.053±0.002 **1**
L50S-S188N-I381V-Δ-N513K	2	0.018±0.131 **63**	0.005±0.004 **0.3**	0.319±0.163 **4**	0.012±0.01 **0.2**
L50S-S188N-I381V-Δ-N513K[1]	21	0.646±0.060 **225**	0.110±0.028 **7**	8.30±1.07 **115**	0.128±0.015 **2**
L50S-S188N-I381V-Δ-N513K[2]	4	1.99±0.248 **693**	0.493±0.080 **30**	18.6±2.3 **259**	0.533±0.050 **10**
L50S-D134G-V136A-I381V-Y461H	26	0.458±0.036 **159**	0.078±0.011 **5**	2.37±0.60 **33**	0.115±0.013 **2**
L50S-D134G-V136A-I381V-Y461H[3]	1	5.53 **1923**	>5 **>305**	>40 **>556**	0.966 **18**

[a]N is number of isolates; [1]CYP51 variant [L50S-S188N-I381V-Δ-N513K] with 120 bp *CYP51* promoter insert; [2]Tolnaphtate insensitive CYP51 variant [L50S-S188N-I381V-Δ-N513K] with both 120 bp *CYP51* promoter insert and 519 bp *MgMFS1* promoter insert; [32]Tolnaphtate insensitive CYP51 variant [L50S-D134G-V136A-I381V-Y461H] with 519 bp *MgMFS1* promoter insert.

(ranging from 2 to 5 ppm). Only one strain of population FR-1 carried S524T but this was in variant [D107-I381V-N513K-S524T]. S524T was more frequently found in the UK-1 and GE-1 populations with 4 and 3 strains, respectively. The most epoxiconazole insensitive strain was found in the GE-1 population; an *MgMFS1* overexpressing strain carrying CYP51 variant [L50S-D134G-V136A-I381V-Y461H-S524T] and producing an EC_{50} value of approximately 10 ppm. Two strains, V6-9A, an overexpressing CYP51 variant [L50S-S188N-I381V-Δ-N513K] from population UK-2, and V9-C23, a CYP51 variant [L50S-V136A-S188N-A379G-I381V-Δ-S524T from population UK-3, showing insensitivity to bixafen but not able to grow in the presence of 5 ppm tolnaphtate were further characterised. Sequencing of the *SdhB*, *C* and *D* revealed multiple target-site alterations in SdhC for both strains (Table 4). No alterations were detected in the SdhB and D subunits. Additional fungicide sensitivity testing revealed that lower levels of SDHI insensitivity were associated with these mutations in comparison with C-H152R.

Table 4 SDHI sensitivity testing of field strains carrying Sdh alterations.

Strains	**Sdh mutations**	**Boscalid sensitivity**	**Bixafen sensitivity**	**Fluxapyroxad sensitivity**
IPO323	None	0.158	0.044	0.020
V6A-9	C-N33T, C-N34T, C-T79N, C-V128M	1.80	0.304	0.644
V9C-23	C-N33T, C-N34T, C-I161S	0.695	0.364	0.472
DP-H152R[1]	C-H152R	3.79	1.24	2.48

[1]Strain isolated in Devon (UK) during 2015 (kindly provided by DuPont)

UK field strains with Sdh alterations affecting SDHI sensitivity have not been found before in our monitoring studies. However, four different *Sdh* mutations in *Z. tritici* field isolates (B-N225T, C-T79N, C-W80S or C-N86S) with low resistance factors to SDHIs were already

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reported by FRAC at low frequencies in France, Germany, Ireland and the UK before 2015. Control of SLB has not been affected so far (FRAC 2014). This may change as field strains carrying SdhC-H152R, showing high resistance factors to SDHIs *in vitro*, have also recently been detected in Ireland (Dooley et *al.* 2016) and the UK (late season 2015 strains, Fraaije unpublished). Preliminary glasshouse studies have shown that some C-H152R strains cannot be controlled using a full rate of a solo SDHI, so further monitoring studies are needed to establish if these strains are further accumulating in UK field populations or if a fitness penalty is associated with this Sdh variant. The Sdh alterations in the UK seem to evolve in azole insensitive CYP51 variants, indicating that resistance management strategies have been followed but that an additional mixing partner such as chlorothalonil is needed to further delay the spread of strains insensitive to both SDHI and azole fungicides.

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REFERENCES

Cools HJ; Bayon C; Atkins S; Lucas JA; Fraaije BA (2012). Overexpression of the sterol 14α-demethylase gene (*MgCYP51*) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Management Science* 68, 1034-1040.

Cools HJ; Fraaije BA (2013). Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest Managem. Science* 69, 150-155.

Dooley H; Shaw MW; Mehenni-Ciz J; Spink J; Kildea S (2016). Detection of *Zymoseptoria tritici* SDHI-insensitive field isolates carrying the *SdhC*-H152R and *SdhD*-R47W substitutions. *Pest Management Science* doi:10.1002/ps.4269

Dubos T; Pasquiali M; Pogoda F; Casanova A; Hoffmann L; Beyer M (2013). Differences between the succinate dehydrogenase sequences of isopyrazam sensitive *Zymoseptoria tritici* and insensitive *Fusarium graminearum* strains. *Pesticide Biochemistry and Physiology* 195, 28-35.

Fraaije, B.A., Bayon, C., Atkins, S., Cools, H.J., Lucas, J.A., Fraaije, M.W. (2012). Risk assessment studies on succinate dehydrogenase inhibitors, the new weapons in the battle to control Septoria leaf blotch in wheat. *Molecular Plant Patholo*gy 13, 263-275.

FRAC (2014). Minutes of the 2014 SDHI meeting, recommendations for 2015. Omrane S; Sghyer H; Audéon C; Lanen C; Duplaix C; Walker AS; Fillinger S (2015). Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates. *Environmental Microbiology* 17, 2805-2823.

Roohparvar R; De Waard M; Kema G; Zwiers L (2007). MgMfs1, a major facilitator superfamily transporter from the fungal wheat pathogen *Mycosphaerella graminicola*, is a strong protectant against natural toxic compounds and fungicides. *Fungal Genetics and Biology* 44, 378-388.