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RESEARCH PAPERS

Fungicide sensitivity of *Mycosphaerella graminicola* Tunisian isolates: the importance of drug transporter genes in the process of fungicide tolerance

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Summary. Seventeen *Mycosphaerella graminicola* isolates from Tunisia and two reference isolates from Europe (St-Q7-2 and IPO323) were examined for sensitivity to azoxystrobin and tebuconazole and for the importance of the drug transporter genes *MgAtr3* (ABC transporter), *MgMfs1* (MFS transporter), *MgSlt2* (MAP Kinase), *MgGpa1* and *MgGpb1* (cyclic AMP) in the process of fungicide tolerance. All Tunisian isolates were sensitive to both fungicides, but considerable variability in sensitivity, and evidence for slight multidrug resistance toward both fungicides (r = 0.58), were observed. A gene expression assay revealed that *MgAtr3* and *MgMfs1* are involved in tolerance to both fungicides. *MgAtr3* is likely involved in tolerance to tebuconazole, while *MgMfs1* is likely required for tolerance to azoxystrobin. The other genes examined were found more likely to be pathogenicity factors rather than fungicide tolerance factors, except for *MgSlt2* which was weakly induced by azoxystrobin treatment. This study has indicated that the Tunisian population of *M. graminicola* remains more sensitive to strobilurin and azole fungicides than European populations, and reports the importance of the ABC and MFS transporters *MgAtr3* and *MgMfs1* in the mechanism of fungicide tolerance.

Key words: ABC transporters, MFS transporters, azoles, strobilurins.

Introduction

Mycosphaerella graminicola (anamorph: *Zymoseptoria tritici*), causing Septoria tritici blotch (STB), is currently one of the most important foliar diseases of wheat worldwide. Severe STB epidemics can induce 30 to 53% yield losses (Palmer and Skinner, 2002). *Mycosphaerella graminicola* is a haploid heterothallic ascomycete which undergoes both asexual and sexual reproduction. The coexistence in Tunisia of both mating types of *M. graminicola* at the leaf scale has been observed (L. Somai-Jemmali, *unpublished data*), and Boukef *et al*. (2012) reported that the two mating types are equally distributed throughout the country. Boukef *et al*. (2012) concluded that *M. graminicola* was highly diverse and that sexual reproduction was common in Tunisia.

Since host resistance against *M. graminicola* is not completely effective in most wheat cultivars, disease control relies mainly on the use of fungicides. Demethylation inhibitors (DMIs) were the most widely used fungicides against *M. graminicola* until the introduction of strobilurins in 1996. DMIs inhibit sterol 14-alpha-demethylase involved in the biosynthesis of ergosterol, an important component of fungal cell membranes, while strobilurins inhibit mitochondrial

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respiration by binding to the cytochrome bc_1 enzyme complex required for ATP synthesis (Gisi *et al*., 2002; Leroux *et al*., 2007). Due to the intensive use of strobilurins since their introduction, resistance to these fungicides quickly developed in European populations of *M. graminicola*, and then rapidly spread to different geographical regions of Europe (Torriani *et al*., 2009; Siah *et al*., 2010). In Tunisia, recent investigations have reported that the Tunisian population of *M. graminicola* is sensitive to strobilurins (Boukef *et al*., 2012; Stammler *et al*., 2012). DMI fungicides are still effective in Europe against *M. graminicola* as only a shift in the fungal sensitivity has been observed (Stammler *et al*., 2008; Leroux *et al*., 2011). Reduced sensitivity to DMIs is conferred by several mechanisms such as development of point mutations in the *Cyp51* gene encoding the sterol 14-alpha-demethylase, overexpression of *Cyp51* and reduction of DMI accumulation in fungal cells through up-regulation of active efflux proteins. This last mechanism involving drug transporters, such as the ATP-binding cassette (ABC) and/or major facilitator superfamily (MFS) transporters, can mediate multidrug resistance to various classes of fungicides (Leroux *et al*., 2011). ABC and MFS transporters are the two most extensively studied families of transporters involved in efflux (Coleman and Mylonakis, 2009). Fungi dedicate a large proportion of their genomes to encoding transporters. There are approximately 10 to 30 genes encoding transporters for each mega base of genomic DNA in fungal genomes (Ren *et al*., 2007).

The sensitivity of *M. graminicola* populations to DMIs is classified into different resistant types (R-types). Each R-type is associated with amino acid alterations (substitutions and/or deletions) in the Cyp51 enzyme. For instance, genotypes R2, R3 and R5 are associated with substitutions G460D, Y137F and V136A, respectively, while genotype R4 is characterized by Y461S/H or ΔY459/G460 substitutions (Leroux *et al*., 2007; Selim, 2009). Genotypes R6, R7- and R7+ possess I381V, in combination with either Y459S/D/N or Y461S/H (R6), or ΔY459/G460 with A379G (R7+) or without A379G (R7-) (Leroux *et al*., 2007; Stammler *et al*., 2008; Selim, 2009). To date, 22 different amino acid alterations have been identified in the Cyp51 enzyme of Western European *M. graminicola* populations (Cools *et al*., 2011).

The aim of the present study was to investigate multidrug resistance toward azoxystrobin and tebuconazole in *M. graminicola* isolates from Tunisia. First, baseline sensitivity to both fungicides was assessed for a collection of 17 Tunisian isolates sampled in 2008 and for two reference isolates from Europe, St-Q7-2 and IPO323. In addition, the G143A substitution and azole R-types were screened for six isolates differing in their fungicide sensitivity patterns. One isolate (St-08-3), the most tolerant to both fungicides, was subsequently selected to investigate, using reverse-transcriptase PCR (RT-PCR), the importance of five drug transporter genes in the process of fungicide tolerance: *MgAtr3* (ABC transporter), *MgMfs1* (MFS transporter), *MgSlt2* (MAP Kinase), *MgGpa1* and *MgGpb1* (cyclic AMP). Most of these genes (except for *MgMfs1* and *MgSlt2*) have previously been studied only for their involvement in fungal pathogenicity, and have not been examined for their involvement in multidrug resistance toward azoxystrobin (except *MgMfs1*) and tebuconazole.

Materials and methods

Fungal isolation and inoculum production

Seventeen *M. graminicola* isolates collected from different regions of Tunisia in 2008 were used in this study (Table 1). Two additional isolates from Europe (St-Q7-2 from Germany and IPO323 from the Netherlands) were used as reference. To obtain the isolates, wheat leaves infected with STB were attached to moist filter paper and incubated in a damp environment at 18°C for 24 h to induce extrusion of cirri from pycnidia. Individual cirri were picked off and transferred to fresh potato dextrose agar (PDA) medium. After incubation for 7 d at 20°C, cultures obtained were transferred onto new PDA medium.

Inoculum was produced by cultivating the isolates in liquid yeast-sucrose medium (YES: yeast extract 10 g L^{-1}, sucrose 10 g L^{-1}) for 7 d at 20°C on a rotary shaker at 100 rpm. Spores were collected by centrifugation at 5000 g for 5 min and washed twice with sterile distilled water. Spore suspensions were adjusted to 10^5 spores mL^{-1} for *in vitro* spore germination assays, and to 10^7 spores mL^{-1} for field inoculation.

Fungicide sensitivity bioassays

In vitro spore germination assay

The effects of azoxystrobin and tebuconazole on spore germination of each isolate was carried out on solid medium (10 g L^{-1} glucose; 12.5 g L^{-1} agar;

Table 1. *Mycosphaerella graminicola* isolates from regions of Tunisia, plus two reference isolates, with their half maximal inhibitory concentration (IC_{50}) values for azoxystrobin and tebuconazole sensitivity based on a spore germination assay.

Strain	Regional origin	Host species	IC_{50} (mg L^{-1}) azoxystrobin	IC_{50} (mg L^{-1}) tebuconazole
St-08-6	Northwest	Durum wheat	0.008	0.013
St-08-48	North	Durum wheat	0.009	0.011
St-08-12	North	Durum wheat	0.013	0.039
St-08-31	North	Durum wheat	0.015	0.039
St-08-53	North	Durum wheat	0.016	0.212
St-08-4	North	Durum wheat	0.022	0.010
St-08-30	North	Durum wheat	0.023	0.013
St-08-5	North	Durum wheat	0.025	0.008
St-08-63	North	Durum wheat	0.032	0.009
St-08-1	North	Durum wheat	0.047	0.012
St-08-29	Middle	Durum wheat	0.052	0.064
St-08-21	North	Durum wheat	0.082	0.015
St-08-2	North -west	Durum wheat	0.097	0.011
St-08-34	North	Durum wheat	0.156	0.010
St-08-46	North	Durum wheat	0.224	0.040
St-08-73	Middle	Durum wheat	0.484	0.095
St-08-3	North	Durum wheat	0.650	0.206
IPO323	Netherlands	Bread Wheat	0.232	0.022
St-Q7-2	Germany	Bread Wheat	> 5	> 5

K_2HPO_4 2 g L^{-1}; KH_2PO_4 2 g L^{-1}) amended with different concentrations of either of the two fungicides. Concentrations used for azoxystrobin were 0.0032, 0.016, 0.08, 0.4, 2, 5 or 10 ppm, while those for tebuconazole were 0.0025, 0.025, 0.25, 1, 2.5, 3.5 or 5 ppm. Both fungicides were added to the medium after autoclaving. Aliquots of 10^5 spores mL^{-1} of each isolate were sprayed on fungicide amended media in Petri dishes. Inoculated Petri dishes were incubated at 20°C in the dark for 2 d. Three dishes were used as replicates for each treatment. The percentage of germinated spores calculated from 100 spores was determined for each dish using a light microscope.

Field assay

A trial was carried out during the 2008/2009 growing season to assess the curative activity of azoxystrobin and tebuconazole against STB under field conditions. The assay was undertaken using *M. graminicola* isolate St-08-3, which was found to be tolerant to both fungicides in the *in vitro* bioassay. The trial was conducted in 1 m^2 plots sown with the susceptible durum wheat cv. Karim (380 seeds m^{-2}), using supplementary irrigation. Plants were inoculated using a backpack sprayer at Zadoks GS 37 with a spore suspension of 10^7 spores mL^{-1}. At 20 days post-inoculation (dpi), corresponding to Zadoks GS 45, the plants were treated using a backpack sprayer with 1 L solution containing azoxystrobin and tebuconazole commercial fungicides at 300 mg L^{-1}. Three plots were used as replicates for each treatment. Fungicide efficacy was evaluated by comparing disease severity at 14, 21, 28, 35, 42, 49, 56, 63 and 70 dpi with that of control plants treated with water.

Fungal DNA extraction

Mycosphaerella graminicola isolates were grown in YES medium for 5 d at 20°C. Spores were collected by centrifugation at 5000 g for 5 min and washed twice with sterile distilled water. Approximately 300 mg of dried fungal biomass was ground in liquid nitrogen and then transferred into a sterile Eppendorf tube to which 500 µL of extraction buffer (EDTA 0.05 M, Tris 0.1 M at pH 8.0, NaCl 0.5 M, 0.7% β-mercaptoethanol and 0.25% SDS) were added. The suspension was incubated for 1 h at 65°C and mixed by inversion every 15 min. The supernatant obtained after centrifugation (10 min, 13000 rpm) was transferred to a new tube and 1/3 V/V of NaOAC 5 M added. The mixture was placed on ice for 20 min. After 10 min of centrifugation at 13000 rpm, the supernatant was transferred into another Eppendorf tube to which 10 µg μL^{-1} RNase solution was added. The mixture was incubated at 37°C for 30 min. An equal volume of phenol-chloroform was added and the mix was centrifuged for 1 min at 13000 g. The watery phase was then extracted twice with 1 V/V chloroform. To precipitate DNA, 1 V/V cold isopropanol was added to the supernatant and the tubes were kept at -20°C for 1 h. The tubes were centrifuged at 13,000 g for 10 min at 4°C. The DNA pellet was then washed with 70% cold ethanol and centrifuged at 13000 g for 5 min. The pellet was dried overnight in a flow-cabinet and then suspended in 1× TE buffer (Tris-HCl 10 mM pH8, EDTA 1 mM pH8). The DNA of each isolate was stored at -20°C until used. DNA was quantified at 260 nm (Cary 50 UV–Vis spectrophotometer; Varian, France) and the concentrations were adjusted to 10 ng μL^{-1} for PCR reactions.

Determination of G143A substitution

The G143A substitution conferring resistance to strobilurins was determined using a mismatch amplification mutation assay on part of the cytochrome *b* gene (Ware, 2006; Siah *et al*., 2010). Primers used were those described previously by Ware (2006). The PCR was performed in a 20 µL reaction mix containing 5× PCR reaction buffer (green GoTaq, Promega), 2 mM dNTP, 10 µM of each primer, 0.5 unit of *Taq* polymerase and 20 ng of template DNA. PCR thermal conditions consisted of an initial denaturation step at 94°C for 5 min followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and then terminated at 72°C for 7 min. PCR products were separated by electrophoresis on ethidium-bromide stained 1.5 % agarose gels run in 0.5× Tris-acetic acid-EDTA buffer at 100 V for 45 min. Gels were visualised under UV light.

Determination of azole R-types

Characterization of isolate azole R-types was performed using quantitative PCR assay according to Selim (2009). 'Low resistant' (LR) genotypes containing isoleucine at position 381 (I381) of the Cyp51 enzyme were determined with TaqMan quantitative PCR, using primers and probes described by Selim (2009). The TaqMan duplex assay was carried out in 25 µL reaction mixture containing 12.5 µL Universal TaqMan PCR Master Mix (Applied Biosystems, Foster City, USA), 0.3 µM of each primer, 0.2 µM of each probe and 50 ng of template DNA. Thermal conditions for quantitative PCR were: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Quantitative PCR assay was carried out using an ABI PRISM 7300 sequence detection system (Applied Biosystems). Standard curves for each target were generated by plotting known amounts of DNA against cycle threshold (Ct) values.

Determination of SNPs associated with R2, R3, R5, R6, R7- and R7+ R-types was carried out with SYBR Green quantitative PCR, using primers reported by Selim (2009). PCR reaction was performed in a 25 µL mixture containing 12.5 µL of SYBR Green PCR master mix (Applied Biosystems), 0.3 µL of each primer and 50 ng of template DNA. Conditions for quantitative PCR were performed according to Selim *et al*. (2007).

Fungal gene expression

Sampling procedure for RT-PCR

Fungal gene expression was investigated on plants from the field trial. Leaves of control plants (inoculated with *M. graminicola* isolate St-08-3 or treated with water) and from plants inoculated with St-08-3 and treated with azoxystrobin or tebuconazole were harvested at 5, 10 and 20 days post-treatment. Sampling was undertaken by cutting off the wheat leaves with scissors. Leaves were placed in aluminum paper, frozen immediately in liquid nitrogen, and stored at -80°C for RNA extraction.

RNA extraction, cDNA synthesis and PCR reaction

The sampled wheat leaves were ground to fine powder in liquid nitrogen using a sterile mortar and pestle. Three leaves from different plants were used as independent replicates for each treatment. Approximately 100 mg of ground leaf powder of each sample was transferred into a sterile 1.5 mL microcentrifuge tube. RNA was extracted using RNA Easy Mini Kit (Invitrogen) according to the manufacturer's protocol. DNase treatment designed to minimize genomic DNA was applied for 40 min. The RNA concentration was measured with a UV spectrophotometer by measuring UV absorption at 260 nm. cDNA synthesis was performed in a mixture of 1× reaction buffer, 2 mM dNTP and 2 μg total RNA in a final volume of 15 μL. Samples were heated at 70°C for 5 min and rapidly chilled on ice before adding 25 units RNase inhibitor (Promega) and 200 units M-MTV reverse transcriptase (Promega). The final mixture was incubated at 37°C for 90 min and then heated at 75°C for 5 min.

PCR reactions were also conducted to amplify five *M. graminicola* target genes (*MgMfs1*, *MgAtr3*, *MgSlt2*, *MgGpa1* and *MgGpb1*) and two reference genes (cytochrome b and β-tubulin) from the fungus and the host, respectively. The sequences of these genes are available on the NCBI website, respectively under GenBank accession numbers DQ661911, AF364105, DQ062121, DQ458049, DQ458052, AY247413 and AAD10487. Specific forward and reverse primers were designed for each gene using Bacon Design software and synthesized by Invitrogen Biotechnology (Table 2). PCR was carried out in a 20 μL reaction mixture containing 1× PCR buffer, 2 mM dNTPs, 10 μM of each primer, 0.5 unit of *Taq* polymerase and 20 ng of cDNA using the following protocol: 94°C for 5 min, followed by 30 cycles each with 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCR reaction was terminated at 72°C for 7 min. PCR products were analyzed by electrophoresis on ethidium-bromide stained 1.5% agarose gels run in 0.5× Tris-acetic acid-EDTA buffer at 100 V for 45 min. Gels were visualized under UV light.

Table 2. List of primers used for RT-PCR analysis.

Primer	Sequence (5'-3')
Slt2-F	CAGAGGAGAACGCAGGATAC
Slt2-R	CGGAACAGAGTGTGGAAGG
Atr3-F	GTTCGTCTTCGTCTTCTAC
Atr3-R	GATAGTTCTCACCTTGTCG
Mfs1-F	GTGTTGTGTATGGCGTTGG
Mfs1-R	TGAGGTAGATGGTCTTGATGG
Gpa1-F	GTCGGTCAAGCCATCAAG
Gpa1-R	CGGTTCACAGTCTCATCC
Gpb1-F	GTGGCGGTAAGAGTAGAC
Gpb1-R	AGATTGTAGATTGAGCAGATG
Cytb-F	CTGATGATGGCAACCGCATTCTTA
Cytb -R	GCTGTATCGTGTAAAGCTATTAGA
Beta-tub-F	GCCATGTTCAGGAGGAAGG
Beta-tub-R	CTCGGTGAACTCCATCTCGT

Statistical analyses

The azoxystrobin and tebuconazole sensitivity levels for each isolate were determined as half maximal inhibitory concentration (IC_{50}) values calculated from the corresponding dose-response curves using the GraphPad Prism 5 software (Hearne Scientific Software). Correlation between sensitivity to azoxystrobin and sensitivity to tebuconazole within isolates was measured with the Pearson correlation test using the XLSTAT software. Comparisons of means for disease severity in the field trial were performed with the Tukey test at a significance level P=0.05 using XLSTAT.

Results

Fungicide sensitivity baseline of the isolate collection

All Tunisian *M. graminicola* isolates and isolate IPO323 were found to be sensitive both to azoxystrobin and tebuconazole, since their IC_{50} values were much lower than those of the reference resistant isolate St-Q7-2 (Table 1). IC_{50} values of St-Q7-2 exceed 5 mg L^{-1} for both fungicides. However, there were considerable differences in the sensitivity profiles of the Tunisian isolates (Figure 1). IC_{50} values varied from 0.008 to 0.650 mg L^{-1} for azoxystrobin and from 0.008 to 0.212 mg L^{-1} for tebuconazole (Figure 1, Table 1). The most tolerant Tunisian isolate to azoxystrobin was St-08-3, which had an IC_{50} value 81 times greater than the most sensitive isolate, St-08-6. The most tolerant for tebuconazole, St-08-53, had an IC_{50} value 27 times greater than St-08-05, the most sensitive isolate (Table 1).

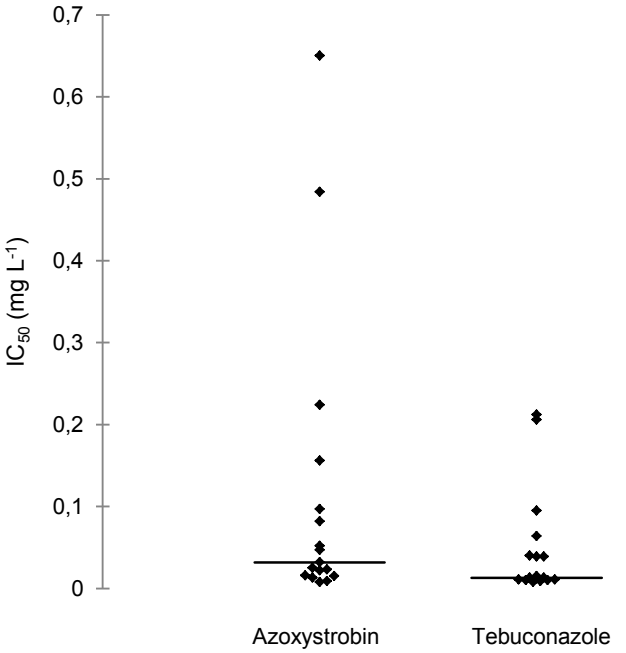

Figure 1. Distribution of IC_{50} values (mg L^{-1}), a measure of fungicide sensitivity, obtained for azoxystrobin and tebuconazole among 17 Tunisian isolates of *Mycosphaerella graminicola*.

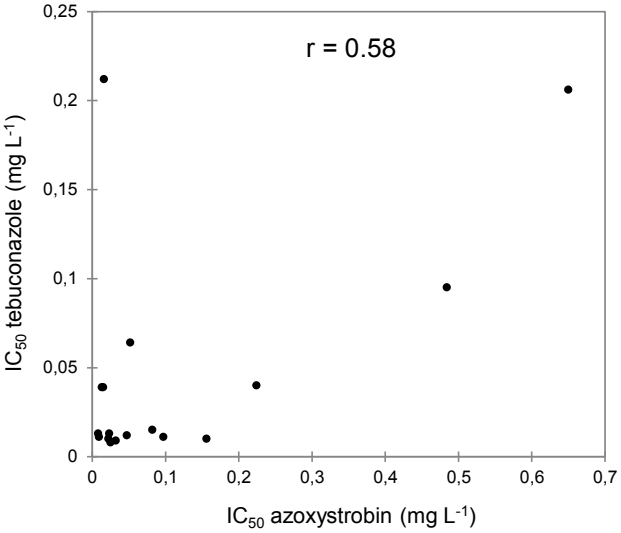

Figure 2. Correlation between sensitivity to azoxystrobin and tebuconazole among 17 Tunisian isolates of *Mycosphaerella graminicola*.

Multidrug resistance to azoxystrobin and tebuconazole

The relationship between sensitivity to azoxystrobin and sensitivity to tebuconazole was tested for Tunisian isolates of *M. graminicola*. A slight positive correlation (r = 0.58) was obtained between sensitivities to both fungicides (Figure 2). Isolates found to be generally tolerant to azoxystrobin (*e.g.* St-08-3) were also tolerant to tebuconazole and isolates found to be generally sensitive to azoxystrobin (*e.g.* St-08-6) were also sensitive to tebuconazole. St-08-53 was an exception and was found to be tolerant to tebuconazole but sensitive to azoxystrobin (Table 1, Figure 2). The majority of the isolates were highly sensitive to both fungicides (Figure 2).

Molecular characterization of six selected isolates with different sensitivity levels

Six *M. graminicola* isolates (St-08-1, St-08-2, St-08-3, St-08-4 and the reference isolates St-Q7-2 and IPO323) with different fungicide sensitivity patterns were subsequently selected for molecular characterization. The mismatch PCR assay performed to screen the G143A substitution revealed that all Tunisian isolates and the reference isolate IPO323 carried the wild type allele G143 conferring sensitivity to azoxystrobin (Figure 3). As expected, the resistant reference isolate St-Q7-2 possessed the resistant allele A143 which confers resistance to azoxystrobin.

The TaqManq PCR assay to determine low resistant genotypes showed that only reference isolate St-Q7-2 was resistant to azoles (Ct = 35.5). The other isolates had Ct values for the I381 allele of <30 indicating they are low resistant (Table 3). Results of the SYBER Green PCR analysis revealed that isolates IPO323 and St-08-4 belong to the R2 type (Ct values <30), while the other isolates belong to either R1 type or R4 type (Ct values >30). According to the IC_{50}s obtained for these isolates with tebuconazole and referring to Leroux *et al.* (2007), we conclude that St-08-1 and St-08-2 belong to the R1 type since their IC_{50}s for tebuconazole were small, while St-08-3 belongs to the R4 type since its IC_{50} for tebuconazole was large (Table 1).

Effect of fungicide application on STB severity

In the field trial, the weekly disease severity assessments showed varied responses by *M. gramini-*

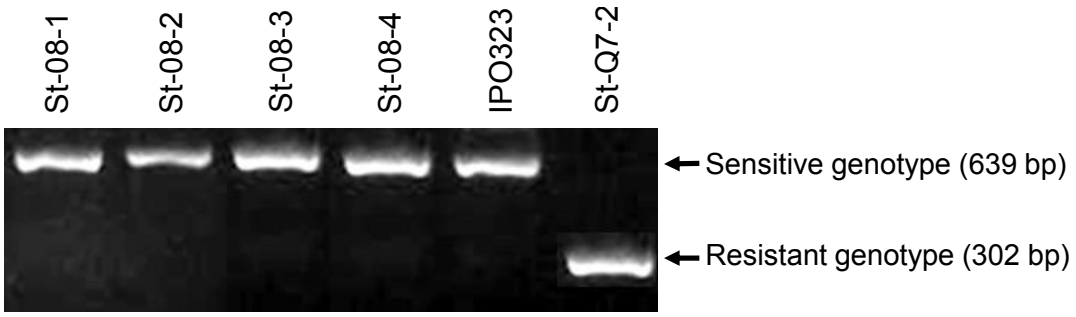

Figure 3. Mismatch PCR assay of G143 or A143 *cytochrome b* alleles in six selected *Mycosphaerella graminicola* isolates. Strobilurin sensitive isolates (Tunisian isolates and IPO323) carrying the wild-type allele G143 show an amplicon of 639 bp. The reference isolate St-Q7-2 carrying the resistant allele A143 shows an amplicon of 302 bp.

Table 3. Cycle threshold (Ct) values obtained with TaqMan and SYBER Green quantitative PCR to determine azole R-types of six selected *Mycosphaerella graminicola* isolates.

Isolate	LR (I381)[a]	R2 (G460D)	R3 (Y137F)	R5 (V136A)	R6 (I381V)	R7- (ΔY459/ G460)	R7+ (A379G)
St-08-1	**19.2**[b]	31.1	39.3	35.4	38.3	39.0	36.3
Tu-08-2	**18.7**	33.6	39.4	39.2	39.2	39.0	39.0
St-08-3	**17.3**	38.4	39.1	39.3	39.1	39.1	39.2
St-08-4	**18.0**	**28.8**	39.3	39.1	37.5	39.0	36.9
IPO323	**19.6**	**28.8**	33.7	32.4	35.1	32.5	36.2
St-Q7-2	35.5	32.1	39.6	35.3	39.0	39.0	39.4

[a] In brackets: *Cyp51* single nucleotide polymorphism(s) corresponding to each azole R-type, screened according to Selim (2009).
[b] In bold: lower Ct values (<30) indicate the presence of the corresponding screened allele.

cola isolate St-08-3 following fungicide application. No differences occurred between control plants and plants treated with azoxystrobin. However, tebuconazole treatment significantly reduced disease severity compared to the control (Figure 4). This reduction was observed relatively quickly after fungicide application i.e. at 35 dpi. At 56 dpi, plants treated with tebuconazole had 26% less STB compared to the control.

Gene expression in *Mycosphaerella graminicola* isolate St-08-3 following fungicide application

The expression pattern of five drug transporter genes (*MgAtr3*, *MgMfs1*, *MgSlt2*, *MgGpa1* and *MgGpb1*) compared to the *cytochrome b* encoding gene (fungal control) was investigated to determine the importance of these genes in the mechanism of fungicide tolerance. This assay was undertaken on field plants inoculated with isolate St-08-3 and treated either with water (control), azoxystrobin or tebuconazole. The expression level of the *cytochrome b* gene in plants treated with azoxystrobin was similar to that obtained for control plants (Figure 5), supporting the phenotypic data showing a lack of efficacy by azoxystrobin against STB development mediated by St-08-3 (Figure 4). In contrast, *cytochrome b* gene expression was totally inhibited at day 20 following treatment with tebuconazole (Figure 5), again reflecting the reduction in STB severity observed in plants treated with this fungicide (Figure 4).

The two drug transporter genes *MgAtr3* and *MgMfs1* were overexpressed after treatment with both fungicides compared to the control (Figure 5). *MgAtr3*

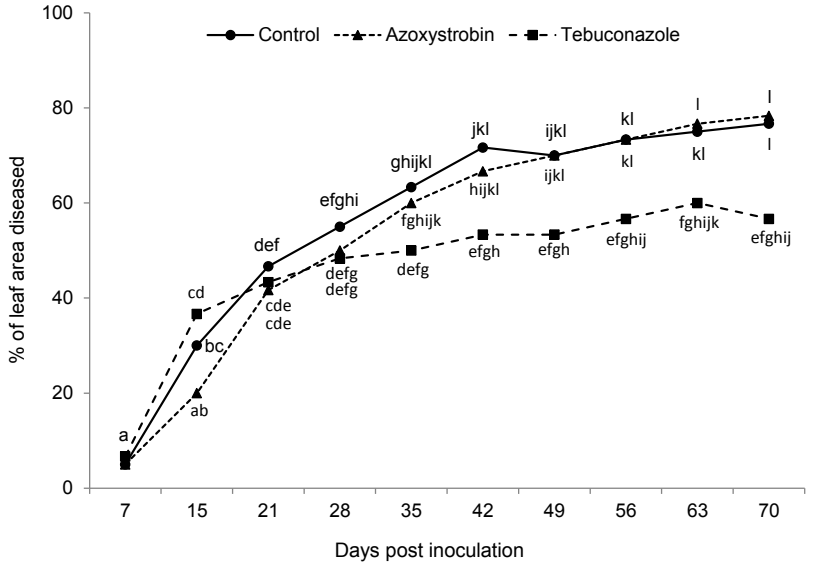

Figure 4. Septoria tritici blotch severity in plants of durum wheat cv. Karim inoculated with Tunisian *Mycosphaerella graminicola* isolate St-08-3 and treated either with water (control), azoxystrobin or tebuconazole fungicide. Means tagged with the same letter are not significantly different using the Tukey test at $P = 0.05$.

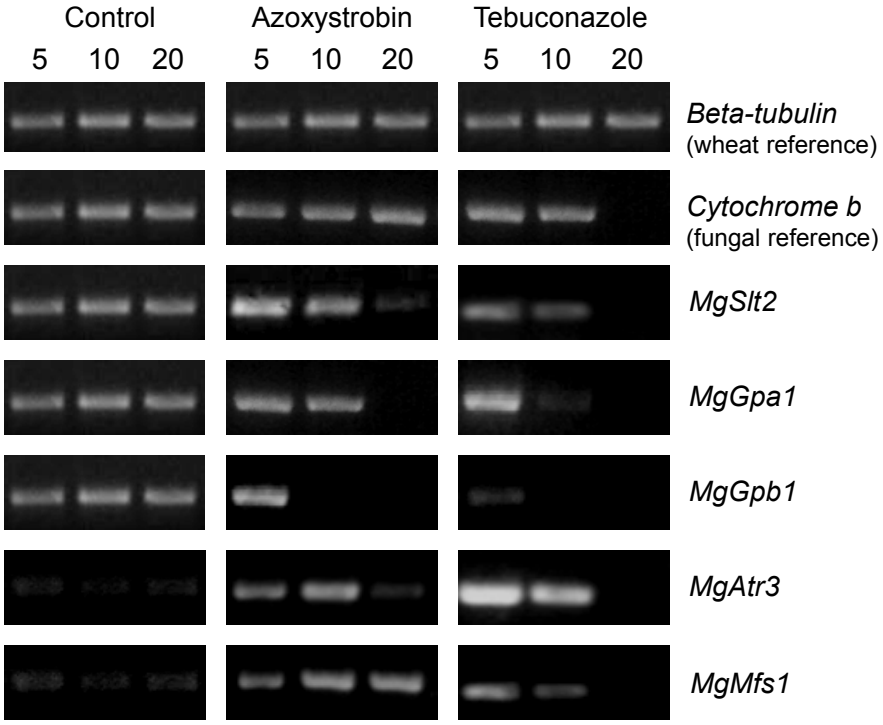

Figure 5. *In planta* RT-PCR expression pattern of five genes (*MgSlt2*, *MgGpa1*, *MgGpb1*, *MgAtr3*, *MgMfs1*) in *Mycosphaerella graminicola* isolate St-08-3 at 5, 10 and 20 days following treatment with water (control), azoxystrobin or tebuconazole fungicide. Days following treatment correspond to 25, 30 and 40 days after inoculation with St-08-3. *Beta-tubulin* and *cytochrome b* used as reference genes for wheat and *M. graminicola*, respectively.

was upregulated at 5 and 10 days after treatment with azoxystrobin or tebuconazole, while the *MgMfs1* gene was up-regulated at 5, 10 and 20 days after treatment with azoxystrobin, and at 5 and 10 days after treatment with tebuconazole. The other genes, *MgSlt2*, *MgGpa1* and *MgGpb1*, were expressed in control as well as in treated plants; *MgSlt2* was overexpressed at 5 days after azoxystrobin treatment and was up-regulated at 20 days. The expression of genes *MgGpa1* and *MgGpb1* decreased after treatment with both fungicides.

Discussion

All 17 *M. graminicola* isolates from Tunisia were found to be sensitive to azoxystrobin and tebuconazole, although their sensitivity levels varied. The molecular screening assay confirmed this finding and showed that only the resistant reference isolate St-Q7-2 from Germany possessed the A143 allele conferring resistance to strobilurins. These results agree with those of Boukef *et al*. (2012) who showed absence of the A143 allele in a population of 357 *M. graminicola* isolates sampled in Tunisia during the 2007/2008 growing season.

Boukef *et al*. (2012) found only two *Cyp51* mutations (G460D and Y461S) associated with reduced azole sensitivity among 80 isolates they assessed. Likewise, Stammler *et al*. (2012), who analyzed 52 Tunisian isolates sampled in 2010, found that all were highly sensitive to pyraclostrobin and epoxiconazole. Among the six isolates screened here for the *Cyp51* gene, only St-Q7-2 was found to be highly tolerant to tebuconazole, whereas the other isolates, including the four Tunisian isolates tested, were found to have low resistance (resistance genotypes R1, R2 or R4). The Tunisian population of *M. graminicola* is therefore different from the European population. Recent investigations have shown that Western European populations of *M. graminicola* are fully resistant to strobilurins (Siah A., unpublished data) and possess increased frequency of multiple drug resistant haplotypes with high levels of reduced azole sensitivity (Stammler *et al*., 2008; Leroux *et al*., 2011). The Tunisian *M. graminicola* population is likely to be more fungicide sensitive because of the less exposure to fungicides in Tunisia than in Europe, where fungicide application is more prevalent.

Our results indicated a slight positive correlation between reduced sensitivity to azoxystrobin and reduced sensitivity to tebuconazole among the isolates we assessed, suggesting the occurrence of multidrug cross-resistance within some isolates, such as St-08-3. This multidrug resistance toward molecules with different modes of action could be explained by the presence in individual isolates of an enhanced active efflux system leading to reduction in the intracellular concentrations of antifungal compounds. This type of mechanism operates in a broad range of fungi and is attributed to the increased activities of membrane drug transporters such as ABC and MFS transporters (Leroux *et al*., 2011).

All this led us to investigate the *in planta* expression pattern of five *M. graminicola* genes, to examine their involvement in the process of fungicide tolerance. Expression of *MgAtr3* and *MgMfs1* was found to be induced by the fungicide treatments, while expression of the other genes occurred both in control and fungicide-treated plants. This indicates that both *MgAtr3* and *MgMfs1* are likely involved in the mechanism of fungicide tolerance, although their expression appears to be fungicide-dependent. Over-expression of *MgAtr3* was more pronounced in plants treated with tebuconazole, while expression of *MgMfs1* was more pronounced following treatment with azoxystrobin, pointing to the importance of MgAtr3 transporter in tolerance to tebuconazole and MgMfs1 transporter in tolerance to azoxystrobin.

This finding could explain why isolate St-08-53 was highly tolerant to tebuconazole but sensitive to azoxystrobin: the isolate may possess high MgAtr3 transporter activity involved with tebuconazole tolerance and low MgMfs1 transporter activity involved with tolerance to azoxystrobin. Based on mutagenesis strategy, the *MgMfs1* gene has been implicated as a major factor in multidrug resistance, especially against strobilurins (Roohparvar *et al*., 2008); this agrees with our results. Other drug transporter genes, such as *MgAtr1*, also have been studied for their role in fungicide tolerance in *M. graminicola*, and this gene was shown to be a determinant factor for tolerance to cyproconazol (Zwiers *et al*., 2002). The ABC transporter gene *BcatrB* has been demonstrated to be a determinant factor for fungicide tolerance in *Botrytis cinerea* (Vermeulen *et al*., 2001). ABC and MFS transporters have also been shown to contribute to fungicide tolerance in *Aspergillus nidulans* (Del Sorbo *et al*., 2000).

Genes *MgSlt2*, *MgGpa1* and *MgGpb1* were expressed in control plants as well as in plants treated with fungicides. This suggests that these genes are

pathogenicity factors which could have greater roles in the fungal infection process, rather than in the mechanisms of fungicide tolerance. Using a gene disruption approach, Mehrabi *et al*. (2006a) demonstrated that *MgSlt2* is essential for spore germination, fungal growth, and host invasion. Likewise, Mehrabi *et al*. (2009) reported that *MgGpa1* and *MgGpb1* play major roles in mycelium melanisation, hyphal development, and anastomosis. Other genes also have been found to be important in *M. graminicola* pathogenesis, and *MgFus3*, for example, was shown to be necessary for mycelium melanisation, host penetration, and pycnidium formation (Cousin *et al.*, 2006), while *MgHog1* was necessary for filamentous growth, dimorphic transition and osmoregulation (Mehrabi *et al.*, 2006b). Similarly, *MgAtr4* was reported to be implicated in host colonization and pycnidium formation (Stergiopoulos *et al*, 2003). We observed a slight overexpression for gene *MgSlt2* compared to the control at day 5 following azoxystrobin treatment, but this decreased over time until inhibition. This slight gene induction by azoxystrobin suggests that *MgSlt2* may play a weak role in reducing *M. graminicola* sensitivity to this fungicide.

Conclusions

The results of the present study indicate that the Tunisian population of *M. graminicola* remains sensitive both to strobilurin and DMI fungicides, in contrast to the European populations which are currently resistant to strobilurins and highly tolerant to some DMIs. However, the Tunisian isolates we assessed showed considerable variability in their levels of sensitivity, and multidrug resistance toward both fungicide classes was demonstrated in some isolates, particularly St-08-3. An *in planta* gene expression assay indicated that the ABC and MFS transporter genes *MgAtr3* and *MgMfs1* contribute to fungicide tolerance in *M. graminicola*. *MgAtr3* is likely to have greater involvement in tebuconazole tolerance, and *MgMfs1* in tolerance to azoxystrobin. The other genes studied, *MgSlt2*, *MgGpa1* and *MgGpb1*, may be considered to be pathogenicity factors but are not likely to be fungicide tolerance factors.

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