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RESEARCH PAPERS

Identification of QoI fungicide-resistant genotypes of the wheat pathogen *Zymoseptoria tritici* in Algeria

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Summary. Septoria tritici blotch caused by *Zymoseptoria tritici* is currently one of the most damaging diseases on bread and durum wheat crops worldwide. A total of 120 monoconidial isolates of this fungus were sampled in 2012 from five distinct geographical locations of Algeria (Guelma, Annaba, Constantine, Skikda and Oran) and assessed for resistance to Quinone outside Inhibitors (QoI), a widely used class of fungicides for the control of fungal diseases of wheat. Resistance was screened using a mismatch PCR assay that identified the G143A mitochondrial cytochrome b substitution associated with QoI resistance. The isolates were QoI-sensitive, since all possessed the G143 wild-type allele, except for three isolates (two from Guelma and one from Annaba), which had fungicide resistance and possessed the A143 resistant allele. QoI resistance was confirmed phenotypically using a microplate bioassay in which the resistant isolates displayed high levels of half-maximal inhibitory azoxystrobin concentrations (IC_{50}s) when compared to sensitive reference isolates. Genetic fingerprinting of all isolates with microsatellite markers revealed that the three resistant isolates were distinct haplotypes, and were are not genetically distinguishable from the sensitive isolates. This study highlights QoI-resistant genotypes of *Z. tritici* in Algeria for the first time, and proposes a management strategy for QoI fungicide application to prevent further spread of resistance across the country or to other areas of Northern Africa.

Key words: *Mycosphaerella graminicola*, G143A substitution.

Introduction

The causal agent of Septoria tritici blotch, *Zymoseptoria tritici* (Desm.) Quaedvlieg & Crous 2011, formerly *Septoria tritici* (teleomorph: *Mycosphaerella graminicola*) (Quaedvlieg *et al*., 2011), is currently one of the most important pathogens of bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L. subsp. *durum*) in many parts of the world, including Algeria. Severe disease epidemics can reduce yields by 35 to 50% (Ponomarenko *et al*., 2011). Since host resistance against this pathogen is not completely effective in most wheat cultivars, disease control relies on the use of fungicides. Benzimidazoles and sterol-demethylation inhibitors have been the most widely used chemical classes to control *Z. tritici* since, respectively, the early and late 1970s. Since their introduction in 1996, strobilurin fungicides, also known as Quinone outside Inhibitors (QoIs), have been intensively used to manage many agricultural fungal pathogens, including *Z. tritici* (Bartlett *et al*., 2002). QoI fungicides inhibit mitochondrial respiration in fungi by binding to the Quinone outside (Qo) binding site of the cytochrome bc1 enzyme complex, thereby blocking electron transfer and halting ATP synthesis (Gisi *et al*., 2002). The success of QoIs was due to their efficacy, and to additional favourable effects on the host plant physiology (Fraaije *et al*., 2003).

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QoI resistance in *Z. tritici* populations arose rapidly in Europe as a consequence of their intensive use (Fraaije *et al*., 2003). The resistance is conferred by a point mutation in the mitochondrial cytochrome b gene, giving rise to a substitution from glycine to alanine at position 143 (G143A) of the cytochrome b enzyme (Fraaije *et al*., 2003). This mutation prevents binding of QoI fungicides to the Qo site allowing fungi to continue mitochondrial respiration. The first QoI resistant isolates of *Z. tritici* were detected in the United Kingdom in 2001 (Fraaije *et al*., 2003). Their frequency rapidly increased after 2001 in different regions of Europe. Frequencies of resistant genotypes within north-European countries such as Denmark, the Republic of Ireland, the United Kingdom, Belgium, northern France and northern Germany (Amand *et al*., 2003; Gisi *et al*., 2005; Leroux *et al*., 2006; McCartney *et al*., 2007; Torriani *et al*., 2009; Siah *et al*. 2010a) were greater compared to south-European areas such as southern France and southern Germany (Gisi *et al*., 2005; Leroux *et al*., 2006; Siah *et al*., 2010a). Such a gradient of resistance distribution was thought to be due to differences in the intensity of QoI use, because of lower disease pressure in the southern Europe compared to more northern regions (Torriani *et al*., 2009; Siah *et al*., 2010a).

Monitoring of fungicide resistance is of particular importance in pest management strategies to ensure effective disease control, and is crucial for understanding the distribution, evolution and impact of resistance in the field. Although QoI resistance in *Z. tritici* is currently widespread throughout Europe, few investigations have been conducted on the status of resistance in other geographical areas such as Africa. Recent reports from Northern Africa revealed an emergence at low frequencies of such resistance in Morocco and Tunisia (Siah *et al*., 2014; Taher *et al*., 2014). However, there is no information available to date regarding QoI resistance in Algeria, where QoI fungicides are used to control wheat pathogens, including *Z. tritici*. The objective of this study were to determine the presence, and if present, the frequency, of occurrence of resistance to QoI fungicides within the Algerian population of *Z. tritici*.

Materials and methods

Fungal sampling and isolation

A set of 120 monoconidial isolates of *Z. tritici* were obtained in 2012 from five distinct geographical locations of Algeria (Table 1). Thirty isolates were collected at each location (15 from bread wheat and 15 from durum wheat), except in Annaba and Oran, where only 15 isolates per location were obtained; resulting in 60 isolates per wheat species (Table 1). The isolates were collected according to the protocol described by Siah *et al*. (2010b), from naturally infected wheat plants untreated with fungicides. Two European isolates, IPO323 (QoI-sensitive), collected in 1981 in The Netherlands (Kema and Silfhout 1997), and T01193 (QoI-resistant), collected in 2009 in France (El Chartouni *et al*., 2012), were used as reference isolates. All isolates were grown on potato dextrose agar (PDA) for 2 weeks and stored at -80°C until further use.

DNA extraction

DNA extraction was carried out using 2-week-old cultures growing on PDA, according to Siah *et al*. (2010a) with a few modifications. Briefly, approx. 50 mg of cirrhus were harvested from cultures of each isolate and transferred into a 1.5 mL capacity Eppendorf tube. After addition of 200 µL of sterile distilled water, each tube was vortexed and sonicated (Deltasonic, France) for 20 min. Four hundred µL of phenolchloroform were added to each tube, which was then vortexed and centrifuged for 5 min at 15,000 g at 4°C (Hettich, Zentrifugen). The aqueous phase was collected and transferred into a clean Eppendorf tube. After addition of 300 µL of chloroform to each tube, the tubes were vortexed and centrifuged at 15,000 g at 4°C for 3 min. This operation was repeated twice. The aqueous phases were

Table 1. Origin and number of Algerian *Zymoseptoria tritici* isolates used in this study.

Location	Number of isolates from durum wheat	Number of isolates from bread wheat
Guelma	15	15
Constantine	15	15
Skikda	15	15
Annaba	15	-
Oran	-	15
Total	60	60

transferred to new clean tubes. Total DNA was precipitated overnight at -20°C after the addition of 10 µL of NaCl (5M) and 500 µL of absolute ethanol to each preparation. After centrifugation for 30 min at 17,500 g at 4°C, the pellets were washed with 70 % ethanol. The tubes were then centrifuged for 15 min at 17,500 g at 4°C before the contents were dried for 20 min in desiccators. DNA was solubilized in each tube by addition of 40 µL of sterile distilled water for 20 min at 37°C, and the DNA concentration was determined at 260 nm using a UV light spectrophotometer (UV Light, Secoman, France). The tubes containing DNA were then stored at -20°C until further use.

PCR mismatch to determine the G143A substitution

Strobilurin sensitivity or resistance among the isolates was determined using a mismatch amplification mutation assay on part of the cytochrome b gene (Ware, 2006; Siah *et al*., 2010a). Primers were designed with a mismatch on the penultimate nucleotide of the 3' end of the forward primer, in which the ultimate nucleotide was at the point mutation position of codon 143 of the cytochrome b gene. The primer set used to amplify a 639 bp DNA fragment in sensitive isolates was the sense primer StrobSNP2fwd (5'-CTTATGGTCAAATGTCTTTATGATG-3', NT 404-428 of the cytochrome b gene) with a mismatch of T instead of G at nucleotide position 427, and an antisense primer StrobSNP1rvs (5'-GGTGACTCAACGTGATAGC-3', NT 1024-1043). The primer set used to amplify a 302 bp DNA fragment in resistant isolates was the antisense primer StrobSNPrcF7 (5'-CAATAAGTTAGTTATAACTGTTGCGG-3', NT 428-453 of the cytochrome b gene with a mismatch of G instead of T at nucleotide 429, and a sense primer StrobSNPrcR1 (5'-CTATGCATTATAACCCTAGCGT-3', NT 152-173). This resulted in a single nucleotide mismatch on the sequence of the sensitive isolates and a double nucleotide mismatch on the sequence of the resistant isolates, and *vice versa*, for each primer set. PCR reactions and PCR-thermal cycling conditions were performed according to Siah *et al*. (2010a). PCR products were separated by electrophoresis on 1.5% agarose gels run at 100 V for 45 min. After ethidium bromide staining and visualizing under UV light, images were captured with a digital camera (Clara Vision, France) and scored manually.

Phenotypic evaluation of fungicide resistance

Phenotypic assays of QoI sensitivity were performed for 28 isolates (23 Algerian isolates with the G143 allele originating from different geographical locations, three Algerian isolates with the A143 allele, and two reference European isolates), in clear and sterile flat-bottomed polystyrene microplates (Iwaki, Asahi techno glass, Japan) with eight rows of 12 wells. Azoxystrobin (Sigma Aldrich) was added to the medium at 50°C following autoclaving and after suspension in 0.5 mL of dimethyl sulfoxide (DMSO) (Merck). Final concentration of DMSO in the medium, including controls without fungicide, did not exceed 0.5 mL L^{-1} of medium. Plate wells were each filled with 150 µL of liquid glucose peptone medium [14.3 g L^{-1} dextrose (VWR), 7.1 g L^{-1} bactopeptone (Difco laboratories) and 1.4 g L^{-1} yeast extract (Merck) amended with azoxystrobin at concentrations 0.001, 0.006, 0.02, 0.1, 0.4, 1.6, 6.2, 25, or 100 mg L^{-1} (final concentrations in 200 µL of medium)]. Aliquots of 50 µL containing 2×10^5 spores mL^{-1}, obtained by washing a 7-d-old fungal culture grown on PDA at 20°C with sterile distilled water, were added to each plate well. Eight wells were used as replicates for each treatment/isolate combination. Non-inoculated medium without fungicide, as well as inoculated medium without fungicide, were used as experimental controls for each isolate. Plates were incubated for 6 d at 20°C in the dark while being shaken at 140 rpm, after which fungal growth was measured using a plate reader (MRX, Dynex technologies) at 405 nm. For each isolate, a dose-response curve and the corresponding 50% inhibitory concentration (IC_{50}) were determined using the GraphPad Prism 5 software (Hearne Scientific Software).

Microsatellite assay

All isolates were subjected to genetic fingerprinting with microsatellite markers to determine whether the resistant isolates were clones or different genotypes (haplotypes) and if they differed genetically from the sensitive isolates. Genetic fingerprinting was carried out using eight microsatellite markers: ST1A4, ST1E3, ST1E7, ST1D7, ST2E4, ST2C10, ST1G7 (Razavi and Hughes, 2004) and AC0007 (Goodwin *et al*. 2007). PCR reactions were each performed in a 20 µL mixture containing 3.2 µL of 25 mM $MgCl_2$, 1.6 µL of 2.5 mM dNTPs, 0.2 µL of 20 µM of each primer, 0.2 unit of Taq polymerase

and 2 µL of its corresponding 10 × reaction buffer (Amphi Tag Gold, Applied Biosystem, Roche), and 2 µL of fungal DNA (final DNA concentration 1 ng μL^{-1}). PCR thermal cycling conditions were carried out according to Goodwin *et al*. (2007) for the marker AC0007, and Razavi and Hughes (2004) for the other markers. PCR products were separated by vertical electrophoresis on urea-polyacrylamide gel, according to El Chartouni *et al*. (2011). Gel colouration and DNA-band revelation were performed using silver nitrate according to Bassam *et al*. (1991). Isolates possessing the same alleles at all loci were considered clones and only a single representative haplotype was used for analyses. The relationship between haplotypes was calculated using multidimensional scaling (MDS) implemented in the program XLSTAT (Addinsoft, France), in order to detect potential genetic divergence among resistant and sensitive haplotypes. In addition to microsatellite analyses, the mating types of resistant isolates were determined according to the protocol described previously by Siah *et al*. (2010b).

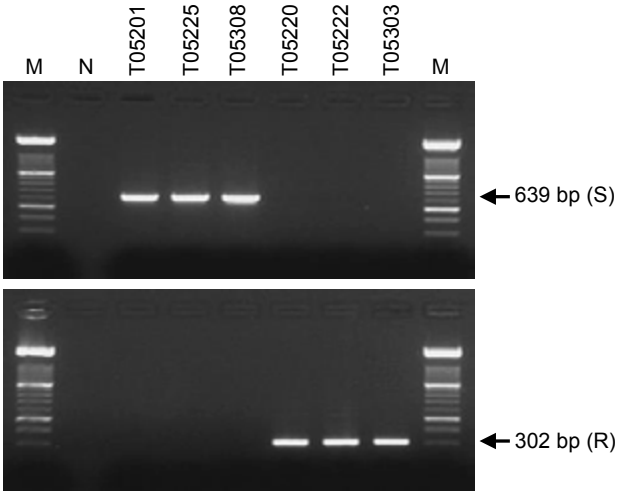

Figure 1. Mismatch amplification mutation assay on a part of the cytochrome b gene to determine the G143 or A143 allele in six Algerian isolates of *Zymoseptoria tritici*. Sensitive isolates carrying the G143 allele generated an amplicon of 639 bp. Resistant isolates carrying A143 allele generated an amplicon of 302 bp. Lane M, molecular size standard XIV (100-bp ladder). Lane N, negative PCR control containing no DNA template.

Results

Detection of *Zymseptoria tritici* haplotypes carrying the A143 resistant allele

All sensitive isolates of *Z. tritici* possessing the G143 wild-type allele generated a single fragment of 639 bp, while the resistant isolates carrying the A143 allele generated a single fragment of 302 bp, as illustrated in Figure 1. Among the assessed isolates, 117 were QoI-sensitive, while three isolates were QoI-resistant, since they possessed the resistant allele A143 (Figure 1). Among the resistant isolates, T05220 and T05222 were from bread wheat from Guelma and T05303 was from durum wheat from Annaba.

Genetic characterization of all isolates with microsatellite markers identified 88 different haplotypes among the 120 assessed isolates, and revealed that the three QoI resistant isolates were different haplotypes since no shared combination of alleles was observed for these isolates (Table 2). MDS analysis showed no genetic differentiation between the resistant and sensitive haplotypes, and overall, all

Table 2. Genetic fingerprinting of the three QoI-resistant Algerian isolates of *Zymoseptoria tritici* with eight microsatellite markers, with their identified mating types. Each letter indicates the allele obtained for each isolate with the corresponding marker.

Isolate	Microsatellite markers								Mating type
	ST1A4	ST1E3	AC0007	ST1E7	ST1D7	ST2E4	ST2C10	ST1G7	
T05220	a	a	a	a	a	a	a	a	MAT1-2
T05222	b	b	b	a	b	a	a	a	MAT1-1
T05303	a	a	a	b	a	a	a	a	MAT1-2

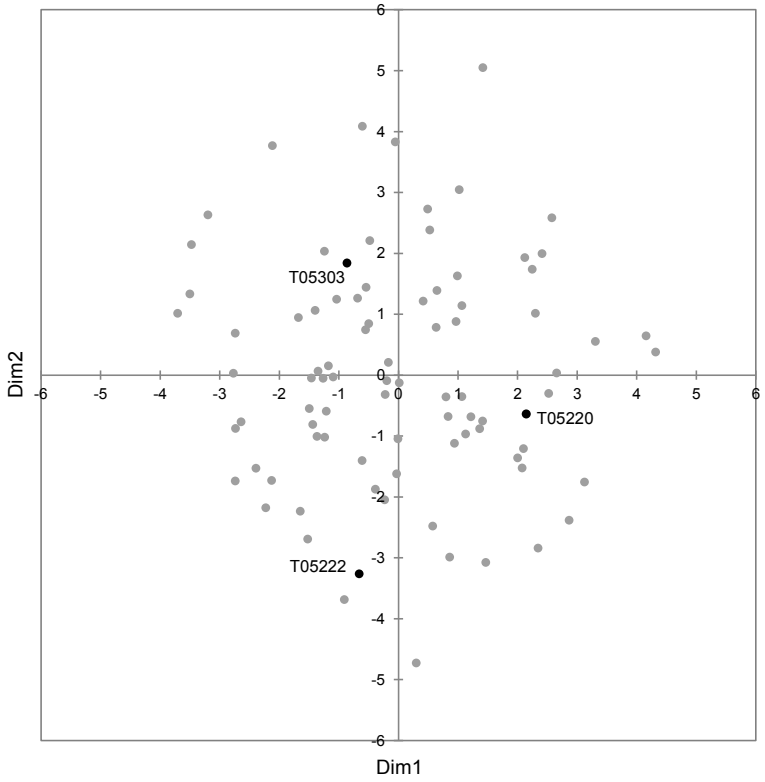

Figure 2. Multidimensional scaling (MDS) of 88 microsatellite-based *Zymoseptoria tritici* haplotypes sampled from five locations in Algeria. Sensitive isolates are presented in clear grey and resistant isolates are presented in black.

of the haplotypes formed a single genetic cluster based on the used microsatellite markers (Figure 2). Regarding the mating type of resistant isolates, both T05220 and T05303 were MAT1-2, whereas T05222 was MAT1-1 (Table 2).

Phenotypic confirmation of resistance

A dose-response curve was determined for each one of the 28 tested isolates from mean values obtained with each concentration of azoxystrobin tested. Sensitive isolates, including the 23 Algerian isolates with the G143 allele plus the sensitive reference isolate from the Netherlands (IPO323), were inhibited by low concentrations of azoxystrobin, as illustrated for four sensitive isolates in Figure 3. The resistant isolates, consisting of the three Algerian resistant isolates with the A143 allele, plus the reference resistant isolate from France (T01193), grew at high concentrations of the fungicide (Figure 3). From these dose-response curves, IC_{50} values were determined for each isolate. All the resistant isolates, including the three from Algeria (T05220, T05222 and T05303) and the French isolate (T01193), displayed high IC_{50} values compared to the sensitive isolates (Table 3), resulting in a distinct bimodal distribution pattern for IC_{50} values (Figure 4). Such a pattern is in agreement with the disruptive (discrete) resistance mode of *Z. tritici* to QoI fungicides. Mean IC_{50} values ranged from 0.001 to 0.09 mg L^{-1} for sensitive isolates and from 7.65 to 61.57 mg L^{-1} for resistant isolates (Table 3).

Frequency and distribution of resistance

The overall frequency of resistant isolates within the sampled population was very low (three out of 120 = 2.5%). The frequency of resistant isolates in Guelma and Annaba was, respectively, 7% (two of 30) and 3% (one of 30). No resistant isolates were found in the other sampled locations (Figure 5). Both Guelma and Annaba are located in eastern Algeria, on the border with Tunisia (Figure 5).

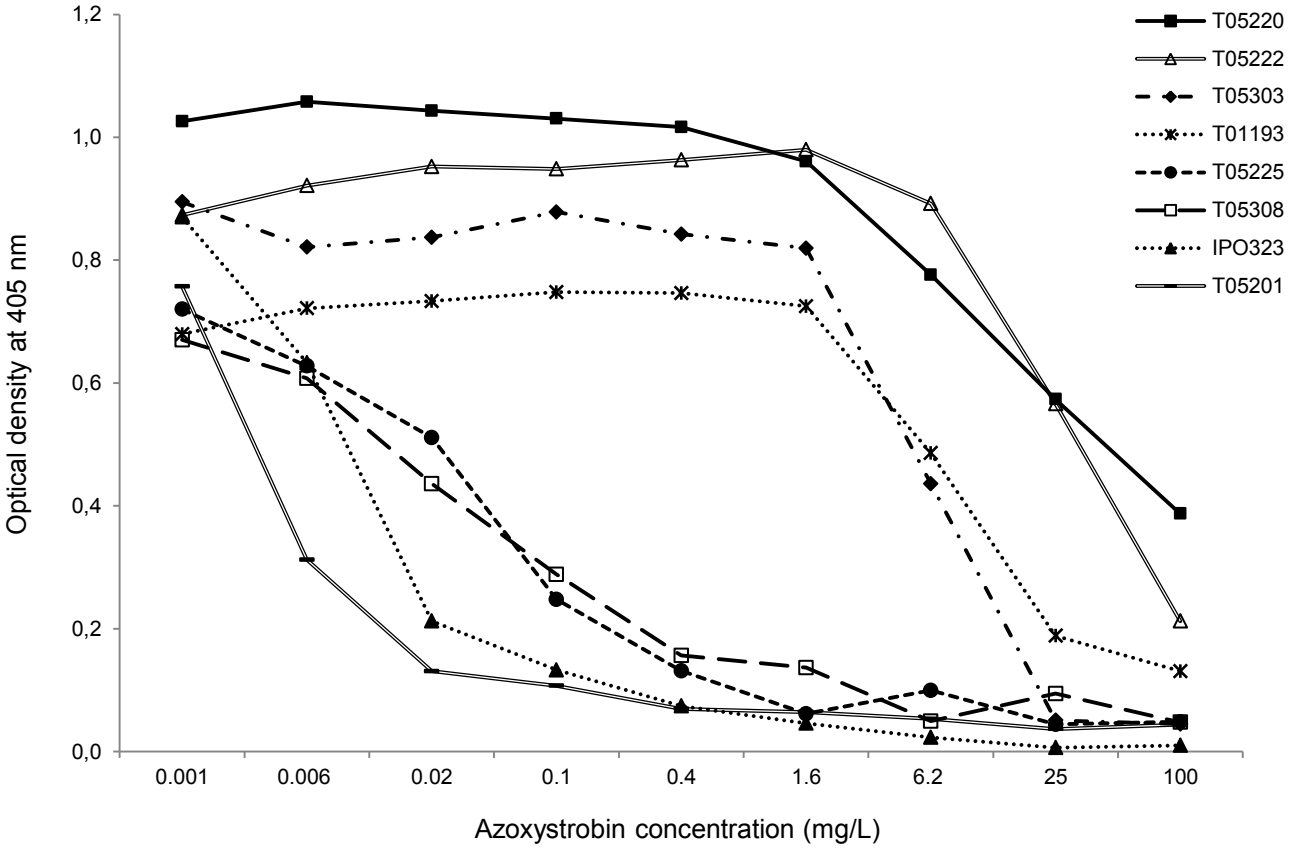

Figure 3. Dose-response curves of six Algerian *Zymoseptoria tritici* isolates and two reference isolates from Europe (IPO323: sensitive; T01193: resistant) obtained with azoxystrobin using a microplate bioassay. T05201, T05225, T05308 are sensitive isolates carrying the G143 allele and T05220, T05222, T05303 are resistant isolates carrying the A143 allele.

Discussion

Monitoring of resistance to single-site fungicides in field pathogen populations is an efficient anti-resistance strategy to ensure effective disease control. In the present study, we detected and confirmed for the first time the occurrence of QoI-resistant genotypes of *Z. tritici* in Algeria, where this class of fungicides is increasingly used in wheat pest management. Two hypotheses may explain this appearance. First, the resistance could be due to local emergence in the field resulting from selection of fungal strains resistant to QoI fungicides, especially in the two Algerian regions of Guelma and Annaba. Secondly, the resistance may have been introduced from Europe through airborne ascospores, since *Z. tritici* ascospores may travel over spatial scales of up to 100 km (McDonald *et al*., 1999). Our results agree with the local emergence hypothesis since no genetic differentiation with microsatellite markers was detected among resistant and sensitive isolates. However, additional genetic investigations are necessary to validate this hypothesis, since no information is available regarding the ability of the markers used to differentiate between Algerian and European haplotypes of the fungus. Further analysis of European and Algerian fungal collections with highly variable microsatellite markers would allow for assessment of levels of recent gene flow (*i.e.* a few generations) and to calculate probabilities of the origin of the resistant Algerian isolates.

Microsatellite fingerprinting revealed that the three resistant isolates are different haplotypes. This indicates that, in the case of local emergence, resistance either arose independently in each one of the three haplotypes or occurred initially in a single haplotype and was then inherited by the other

Table 3. Half maximal azoxystrobin inhibitory concentration (IC_{50}) values for 26 Algerian and two European reference *Zymoseptoria tritici* isolates, established from microplate bioassays.

Isolate	Location	Presence of G143 or A143 allele	IC_{50} (mg L^{-1})	Sensitive (S) or resistant (R)
IPO323	Netherlands	G143	0.008	S
T05201	Guelma	G143	0.001	S
T05207	Guelma	G143	0.02	S
T05211	Guelma	G143	0.01	S
T05216	Guelma	G143	0.07	S
T05221	Guelma	G143	0.07	S
T05224	Guelma	G143	0.09	S
T05225	Guelma	G143	0.04	S
T05231	Constantine	G143	0.07	S
T05233	Constantine	G143	0.006	S
T05234	Constantine	G143	0.02	S
T05252	Constantine	G143	0.01	S
T05283	Skikda	G143	0.08	S
T05284	Skikda	G143	0.08	S
T05286	Skikda	G143	0.06	S
T05396	Skikda	G143	0.07	S
T05300	Annaba	G143	0.09	S
T05302	Annaba	G143	0.04	S
T05305	Annaba	G143	0.01	S
T05308	Annaba	G143	0.04	S
T05309	Oran	G143	0.08	S
T05312	Oran	G143	0.07	S
T05317	Oran	G143	0.08	S
T05319	Oran	G143	0.03	S
T01193	France	A143	11.03	R
T05220	Guelma	A143	12.00	R
T05222	Guelma	A143	61.57	R
T05303	Annaba	A143	7.65	R

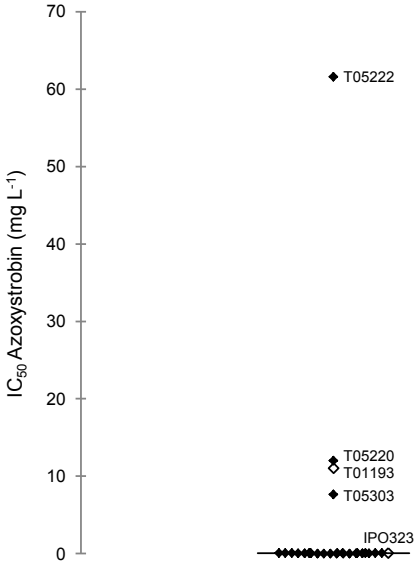

Figure 4. Distribution of azoxystrobin IC_{50} values obtained for 28 *Zymoseptoria tritici* Algerian isolates. Sensitive (IPO323) and resistant (T01193) reference isolates are presented in white.

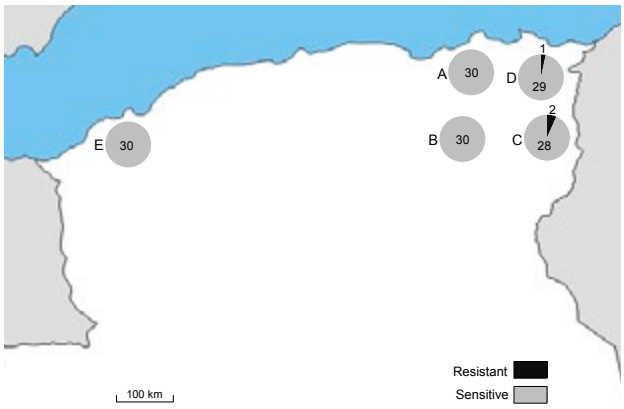

Figure 5. Map of northern Algeria showing the frequencies of QoI-sensitive and QoI-resistant *Zymoseptoria tritici* isolates within the five locations sampled in 2012. Within the circles, sensitive isolates are represented by clear grey and resistant isolates are represented by black. (A: Skikda, B: Constantine, C: Guelma, D: Annaba, E: Oran).

haplotypes *via* sexual recombination, with possible spread of resistance from one location to another *via* wind-borne ascospores. In Europe, two non-exclusive hypotheses have been proposed to explain the rapid spread of QoI-resistance over the continent: firstly, that the G143A mutation occurred only once or a very few times locally and was subsequently

distributed to other regions by migration of resistant isolates; and secondly, that the G143A mutation occurred independently in several different mitochondrial-DNA (mtDNA) haplotypes and/or geographic regions. A study by Torriani *et al*. (2009) with mtDNA sequences, considered the second hypothesis and concluded that the resistance in Europe emerged independently at least four times. The resistant haplotypes then rapidly increased in frequency owing to strong fungicide selection and spread eastward through wind dispersal of ascospores.

The two resistant isolates T05220 and T05222 were from durum wheat, while the other resistant isolate T05303 was from bread wheat. This indicates that fungicide resistant isolates of *Z. tritici* can occur both with bread and durum wheat adapted genotypes. All three resistant isolates were detected in Guelma and Annaba, both regions which are located on the border with Tunisia, where QoI-resistant isolates, at very low frequency, were recently reported (Taher *et al*., 2014). These authors found only three resistant isolates (2%) among 159 *Z. tritici* isolates sampled in Tunisia in 2012, although fungicide resistant isolates were not reported in previous surveys (Boukef *et al*., 2012; Stammler *et al*., 2012; Naouari *et al*., 2013). Likewise, Siah *et al*. (2014) detected nine resistant isolates (9%) in a population of 96 isolates sampled in 2010 in Morocco, while no resistant isolates were found in a collection of 134 isolates obtained in 2008, suggesting recent emergence of resistance in Morocco (Siah *et al*., 2014). The frequencies of QoI resistance in Northern Africa, including Algeria (2.5%), strongly differ from those of Western Europe, where *Z. tritici* populations are currently fully resistant to QoI fungicides (A. Siah, unpublished data). Recent investigations revealed that QoI resistance in *Z. tritici* is also widespread in Eastern Europe, especially in the Czech Republic, where a rapid increase in resistance was observed during the period from 2005 to 2011, coinciding with the widespread application of QoIs (Drabešová *et al*., 2013). The difference between Europe and Northern Africa regarding the level of resistance is likely to be due to lower use of fungicides in Northern African countries compared to Europe. This result indicates that the Mediterranean Sea separating Northern Africa from Europe may constitute a geographical barrier that prevents or limits the migration of resistance by wind-borne ascospores from Europe into Northern Africa. Regarding other parts of the word, no QoI-resistance has been reported in Asia, for instance in Iran, where only the G143 wild-type allele was found among 89 *Z. tritici* isolates sampled from five provinces (Saidi *et al*., 2012). More recently, Estep *et al*. (2013) analyzed two fungal collections from two locations of western Oregon (USA) and found resistant isolates, thereby reporting for the first time the occurrence of QoI-resistant genotypes of *Z. tritici* in America.

In conclusion, this study reports for the first time the presence of QoI-resistant isolates of *Z. tritici* in Algeria. Further genetic investigations should be performed to confirm the local emergence of resistance, or whether the resistance was introduced from Europe *via* wind migration of ascospores (or both). Although resistance is currently (as of 2012) at a low frequency and is locally restricted, an appropriate resistance management strategy is recommended for strobilurin application in Algeria, for example use of these fungicides only in mixtures or in alternation with other classes of anti-fungal agents. This would prevent further spread of fungicide resistance across Algeria or into other parts of Northern Africa.

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