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Prevalence of QoI resistance and mtDNA diversity in the Irish *Zymoseptoria tritici* population

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Abstract

The emergence and spread of Quinone outside Inhibitor (QoI) fungicide resistance in the Irish **Zymoseptoria tritici** ***population in the early 2000s had immediate impacts on the efficacy of the entire group of fungicides for the control of septoria tritici blotch. As a result, a dramatic reduction in the quantities applied to winter wheat occurred in the following seasons. Even in the absence of these fungicides, the frequency of the resistance allele, G143A in the pathogens mtDNA has remained exceptionally high (>97%), and as such, it can be anticipated that continued poor efficacy of current QoI fungicides will be observed. Amongst the isolates with G143A, differences in sensitivity to the QoI pyraclostrobin were observed*** **in vitro**. ***The addition of the alternative oxidase (AOX) inhibitor salicylhydroxamic acid increased sensitivity in these isolates, suggesting some continued impairment of respiration by the QoI fungicides, albeit weak. Interestingly, amongst those tested, the strains from a site with a high frequency of inserts in the*** **MFS1** ***transporter gene known to enhance QoI efflux did not exhibit this increase in sensitivity. A total of 19 mtDNA haplotypes were detected amongst the 2017 strain collection. Phylogenetic analysis confirmed the suggestion of a common ancestry of all the haplotypes, even though three of the haplotypes contained at least one sensitive strain.***

Keywords

AOX • G143A • MFS1 • QoI fungicides • quinone outside inhibitor • SHAM

Introduction

Septoria tritici blotch (STB) caused by the ascomycete *Zymoseptoria tritici* is the most economically destructive disease of winter wheat in temperate climates, such as those that prevail in north-western Europe. Under Irish growing conditions, if left unchecked, STB has the potential to reduce winter wheat yields by upwards of 50% (Burke and Dunne, 2008). Control is achieved through a combination of agronomic practices, varietal resistance and fungicide applications. Unfortunately, the reliability of either agronomic practices or varietal resistance to provide the control required have led to a reliance on fungicides. Currently, Irish winter wheat growers will typically apply fungicides often as mixtures between 3–4 times during the latter stages (growth stages 31–65) of the growing season. Whilst a range of actives are available to the growers in Ireland, with the exception of the multisite fungicides, resistance is now present at varying frequencies in the Irish *Z. tritici* population to the azole, succinate dehydrogenase inhibitors (SDHIs) and the quinone outside inhibitors (QoI) fungicides (Kildea *et al.*, 2010; Kildea *et al.*, 2014; Dooley *et al.*, 2016).

By inhibiting fungal respiration through specific disruption of electron transportation complex III within the target pathogens mitochondria, the QoIs are an extremely effective class of fungicides that have been widely utilised in multiple cropping systems since their commercialisation in the mid-1990s (Gisi *et al.*, 2002). Unfortunately, the specific nature of this activity places the QoIs at a high risk of resistance development. In arable cropping systems, resistance to the QoI developed quite rapidly, initially to *Blumeria graminis* f.sp. *tritici* and *B. graminis* f.sp. *hordei* (cause of mildew in wheat and barley respectively) (Fraaije *et al.*, 2002, 2006), and subsequently, *in Ramularia collo-cygni* (cause of ramularia leaf spot in barley) (Fountaine *et al.*, 2010). Resistance to the QoIs in *Z. tritici* was first detected in 2002, although retrospective resistance testing confirmed its presence in the U.K. population in late 2001 (Fraaije *et al.*, 2005). In the majority of cases, resistance to the QoIs is conferred by the substitution of a glycine by an alanine at position 143 in the cytochrome *b* of the pathogens mitochondria (Gisi *et al.*, 2002). Following its emergence in *Z. tritici*, it rapidly spread through Northern-Europe, dominating populations by 2004. Torriani *et*

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al. (2008) have since confirmed that this was in part due to the fact that G143A emerged separately on at four least occasions throughout the region. In the presence of high frequencies of G143A, field control provided by the QoI is severely impacted, to the point where they are no longer recommended for control of STB (Kildea *et al.*, 2010). As they were principally applied for STB control, the loss of efficacy associated with resistance has resulted in a reduction in their usage on Irish wheat crops, where they now account for approximately 10% of total fungicides applied (Anon, 2014). Similar reductions in usage have occurred elsewhere in Northern-Europe; however, due to the absence of any observable fitness penalties associated with resistance, the frequency of the resistance allele has remained extremely high (Cheval *et al.*, 2017; Heick *et al.*, 2017). In addition to the mutation G143A, multiple drug resistance (MDR) conferred by overexpression of the efflux pump MgMfs1 has been identified as potentially contributing to further reductions in sensitivity to the fungicides, including the QoIs.

As resistance to both the azoles and SDHIs continues to emerge and spread in the Irish population, it is essential to re-evaluate the sensitivity of the contemporary Irish population to the QoIs. In addition to the potential commercialisation of the quinone inside inhibitor (QiI) fenpicoxamid (Inatreq™ active, Corteva Agrisciences; Owen *et al.*, 2017), which also targets cytochrome *b*, albeit at a different location to that of the QoIs, it is imperative to understand the diversity of the mtDNA within Irish *Z. tritici* populations. To achieve these goals, a broad screen of the Irish *Z. tritici* population was conducted in 2016, whilst a more detailed analysis including mtDNA diversity was conducted on three local populations established in 2017.

Materials and methods

Collection, isolation and storage of Z. tritici

Wheat leaf samples showing visible STB symptoms were collected from 65 commercial winter wheat crops in spring 2016 prior to the application of fungicides. All leaves were air dried for a minimum of 48 h, after which they were stored at -20 °C until required. In 2017, three winter wheat crops destined as trial sites in 2017 were sampled in spring prior to the application of fungicides (Table 1). Isolation of *Z. tritici* were performed as described by Dooley *et al.* (2016), with obtained isolates stored at -80 °C in 30% glycerol (v/v). Where possible, a total of ten isolates were retrieved from each of the 2016 samples, with 32 isolates retrieved from each of the 2017 samples.

Table 1. Isolate numbers and genetic parameters of the three 2017 *Zymoseptoria tritici* populations

Location	*n*	S	H	H_d	P_i
Cork	32	13	13	0.847	0.00121
Knockbeg	31	11	9	0.785	0.00117
Meath	32	11	8	0.808	0.00148
Total	95	19	19	0.827	0.00130

n = number of isolates; S = polymorphic sites; H = number of distinct haplotypes; H_d = haplotype diversity; P_i = nucleotide diversity.

QoI sensitivity determination – agar plate assay

Sensitivity of the 2016 isolate collection to the QoI fungicide azoxystrobin was determined using an agar plate assay. Briefly, to create inoculum each isolate was prepared by spotting 30 mL from the glycerol stock stored in -80 °C on un-amended PDA and incubated for 4 days, after which yeast like spores were removed and placed in sterile distilled water (SDW). To create fungicide test plates, the QoI fungicide pyraclostrobin (in its formulated form Modem, BASF) was added to PDA post autoclaving to provide a final concentration of 1 mg/L. In parallel, un-amended PDA was generated to serve as a positive control. Aliquots (30 mL) of each spore suspension were then spotted on to three plates of both the un-amended and QoI amended plates and allowed to air dry. In total, ten isolates were tested per plate, and once dry, the plates were sealed and incubated at 18 °C in the dark for 7 days. After 7 days, the resistance status of each isolate was determined by the presence/absence of growth on both sets of plates.

QoI sensitivity – microtitre plate assay

Sensitivity of the isolates obtained in 2017 to the QoI pyraclostrobin was determined using a microtitre plate assay as described by Dooley *et al.* (2016) with minor adjustments to reflect the test fungicide. This included a reduction in the test concentrations to 0, 0.01, 0.04, 0.123, 0.37, 1.1, 3.3 and 10 mg/L, plus/minus the inclusion of the alternative oxidase inhibitor salicylhydroxamic acid (SHAM) at a concentration of 30 mg/L. Differences in sensitivity between the three sites for both pyraclostrobin ± SHAM were determined by ANOVA using Genstat 18th Edition.

Molecular detection of resistance

Following the QoI sensitivity, screening DNA from all isolates from the 2016 collection failing to grow in the presence of pyraclostrobin and an additional 80 isolates representative of the wider collection was extracted as described by Dooley *et al.* (2016). Similarly, DNA was extracted from all the isolates tested in the 2017 collection. The presence of G143A in these isolates was determined using a PCR-RFLP assay as described by Torriani *et al.* (2008).

Detection of MDR phenotypes

The presence of the three inserts (type I = 519bp; type II = 369/339bp; type III = 149bp) in the promoter of the *MgMfs1* gene previously confirmed to contribute to overexpression of the MgMfs1 efflux pump were determined in the three 2017 collections, as described by Omrane *et al.* (2017).

Determination of mtDNA diversity and phylogeny

Mitochondrial DNA diversity in the *Z. tritici* populations sampled from the three sites in 2017 was determined following sequence analysis of three mtDNA sequences (*Mg1, 2, 3*), as previously described by Torriani *et al.* (2008) and Siah *et al.* (2014). Population genetic analysis (AMOVA and F_{ST} calculations) was conducted as described by Welch *et al.* (2018) using ARLEQUIN 3.5.1.5. A Maximum Likelihood phylogenetic tree was constructed using the concatenated sequence in MEGA 6 and using the Tamura 3-parameter model with a discrete Gamma distribution (five categories).

Results

QoI sensitivity in 2016 and 2017

A total of 503 isolates were successfully isolated and tested for their sensitivity to the QoI fungicide pyraclostrobin in 2016. Of these, only 17 failed to grow in the presence of pyraclostrobin and were confirmed to have the wild-type glycine at position 143 in their cytochrome *b* using the PCR-RFLP assay. Of the additional 80 isolates screened molecularly and which grew in the presence of pyraclostrobin, all had an alanine at position 143 in their cytochrome *b*, confirming their resistance to the QoIs. The sensitive strains were detected in all regions (13 of the 65 sites in 2016), with only one site with >2 of the ten isolates screened confirmed as sensitive.

A total of 96 isolates were tested for their sensitivity to pyraclostrobin from the three sites in 2017 using the microtitre plate assay. Of these, only four isolates were deemed sensitive, confirmed also by the PCR-RFLP assay. Three of these were from the Knockbeg site (Figure 1), whilst the other was from Cork. Amongst the isolates deemed resistant using the PCR-RFLP, a wide range of sensitivity was observed (resistance factors calculated using the sensitive isolate KB8; EC50 0.01 mg/L, ranged from 37–430). Significant differences in the sensitivity (sensitive strains excluded) were observed between sites, with the mean sensitivity (EC50 mg/L) of the population from Knockbeg (1.46 mg/L) significantly less sensitive than either that of Cork (0.99 mg/L) or Meath (0.94 mg/L), with no differences observed between the latter sites (Figure 2) (P = 0.002). The inhibition of alternative oxidase through the addition of SHAM increased the mean sensitivity of the population from both Cork and Knockbeg, but had limited impact on the sensitivity of the population from Meath (Figure 2), resulting in no significant differences in

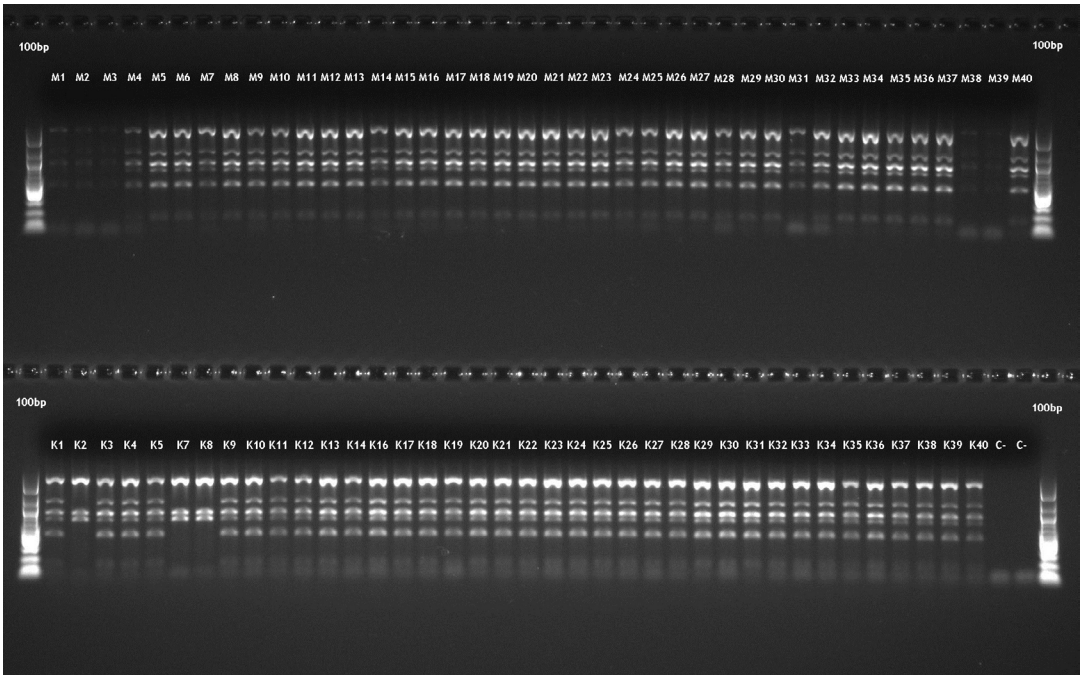

Figure 1. Molecular detection of QoI resistance based on the presence of the G143A mutation. Isolates with the sensitive G143 allele (K2, K7 and K8) are highlighted by the presence of three bands, whilst resistance is detected by an additional site for the restriction enzyme *Fnu*4HI resulting in four distinct bands.

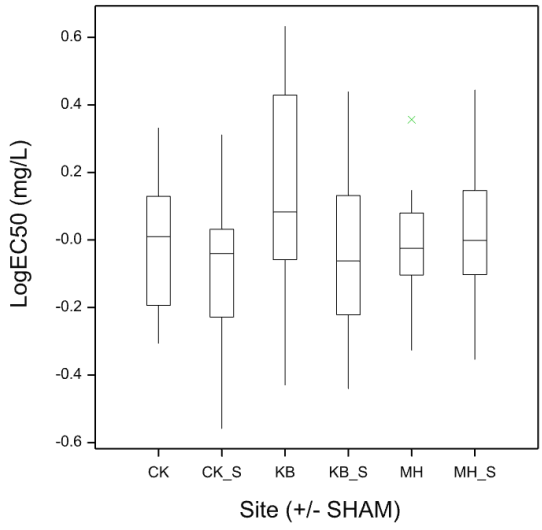

Figure 2. Sensitivity of the three 2017 *Zymoseptoria tritici* populations (CK = Cork; KB = Knockbeg; MH = Meath) to the QoI fungicide pyraclostrobin in the absence and presence of the alternative oxidase inhibitor SHAM (S).

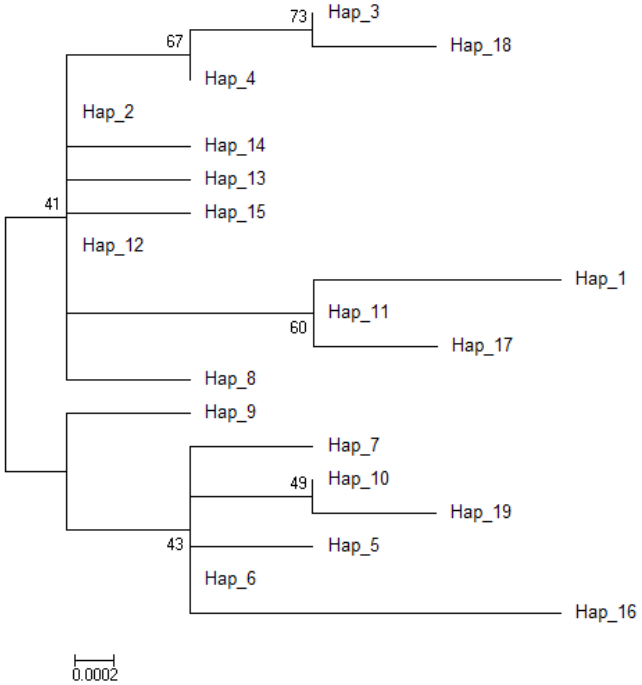

Figure 3. The evolutionary history of the 19 *Zymoseptoria tritici* haplotypes identified amongst the three 2017 populations inferred by using the Maximum Likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6.

sensitivity between the three sites (P = 0.126).

Detection of MDR

Two of the three characterised inserts (I and III) were detected amongst the three populations. Both inserts were detected at a low frequency in both the Cork and Knockbeg collections; 7% insert I and 4% insert III in Cork and 7% and 20% insert III in Knockbeg. In Meath, isolates with insert III dominated the collection, with 60% with insert III. Insert I was not detected in the Meath collection.

MtDNA Diversity

Using the concatenated mtDNA sequences (1,677 bp), a total of 19 haplotypes were identified in the 95 isolates isolated from the three sites in 2017 (a single isolate failed to provide sequence data of sufficient quality) (Table 1). The number of isolates within each haplotype varied from 1–28, with only

Table 2. Distribution of mtDNA haplotypes among the three 2017 *Zymoseptoria tritici* populations

Haplotype	Cork	Knockbeg	Meath	Total
H1	1			*1*
H2[1]	11	12	5	*28*
H3[1]	1	1		*2*
H4	5	3	3	*11*
H5	1			*1*
H6	5	7	10	*22*
H7	1			*1*
H8	2			*2*
H9[1]	1			*1*
H10	1			*1*
H11	1	5	9	*15*
H12	1			*1*
H13	1			*1*
H14		1		*1*
H15		1	2	*3*
H16		1		*1*
H17			1	*1*
H18			1	*1*
H19			1	*1*
Total	*32*	*31*	*32*	*95*

[1]Indicate the presence of QoI sensitive isolates within the haplotype.

Table 3. Population differentiation (F_{ST} above the diagonal) between the three 2017 *Zymoseptoria tritici* populations[1]

Population	Cork	Knockbeg	Meath
Cork		-0.00881	0.03673
Knockbeg	–		-0.00060
Meath	–	–	

[1]F_{ST} above the diagonal; significance at $P < 0.05$ below the diagonal is highlighted as + if present.

four haplotypes common amongst all three sites (Table 2). No significant genetic differentiation was identified amongst the three populations as conferred by low F_{ST} values (Table 3). This was supported by AMOVA, with 99% of the variation within the collection explained by differences within each population, with the remaining 1% due to differences between the populations (Table 4). With the exceptions of Haplotypes 3 and 18, clustering of the different haplotypes into different clades following reconstruction of the maximum likelihood tree was weak, indicating a strong historical backgrounds for the populations analysed (Figure 3).

Discussion

Following the emergence and rapid spread of QoI resistance in *Z. tritici* populations throughout North-western Europe in the early 2000s, control of STB provided by QoI fungicides has been dramatically impacted (Kildea *et al.*, 2010; Blake *et al.*, 2017). This loss of efficacy led to a drop in QoI usage in Irish wheat crops (Anon, 2014). Even with this decrease in usage, resistance has remained extremely high. Such persistence of resistance in the absence of the fungicide indicates the lack of a fitness penalty associated with G143A in *Z. tritici*. Similar persistence of QoI resistance has been detected elsewhere in European Z. tritici populations (Cheval *et al.*, 2017; Heick *et al.*, 2017). Such an apparent lack of a fitness penalty associated with G143A has also been observed in other plant pathogens, although Fisher and Meunier (2008) highlight that differences in fitness do exist and that such differences may be due to additional variations that occur within the cytochrome *b* in these pathogens.

Even though G143A dominated all the collections assessed in the present study, a large range of sensitivity to pyraclostrobin was present in the three populations established in 2017. This variation in sensitivity may contribute to the control still achieved under field conditions by specific QoIs as highlighted by Kildea *et al.* (2010). The increase in sensitivity of the populations from both the Cork and Knockbeg collections to pyraclostrobin once SHAM was added suggests that some of this variation maybe due to differences in alternative oxidase activity within the different collections. If AOX activity is being induced by the QoI, it may indicate that they are still restricting respiration even in the presence of G143A. This has been hypothesised by Kildea *et al.* (2010) as an explanation for the delay in germination of *Z. tritici* pycnidiospores observed in the presence of pyraclostrobin. However, even with the addition of SHAM, a large range of sensitivity was still observed, indicating that additional mechanisms, other than G143A and alternative oxidase activity must be operating in the Irish *Z. tritici* populations. Roohparvar *et al.* (2007, 2008) have previously shown that the expression of the *MgMfs1* gene can contribute to reduced sensitivity to the QoI fungicides. More recently, Omrane *et al.* (2017) have demonstrated the overexpression of this gene in contemporary *Z. tritici* field populations. It is interesting to note that the addition of SHAM had little impact on the sensitivity of the collection from Meath. This collection was dominated by isolates with insert III in their *Mfs1* promoter region as identified by Omrane *et al.* (2017). Whilst this insert has only minor impacts on Mfs1 activity as identified by Omrane *et al.* (2017), it may be sufficient to mask any reduction in alternative oxidase activity resulting from the addition of SHAM.

Although only four of the 19 mtDNA haplotypes identified in the 2017 collection were common across all three populations examined, analysis of the concatenated mtDNA sequence suggested high levels of gene flow between the populations, with almost all of the variation present within the populations and not between. This is in agreement with previous studies involving European and global *Z. tritici* populations (Zhan *et al.*, 2004; Torriani *et al.*, 2008). Phylogenetic analysis of the different haplotypes suggests a common ancestry for the Irish *Z. tritici* populations. This could be a reflection of the selective sweep that occurred following the emergence of resistance; however, as three of the haplotypes included at least one isolate that was sensitive, this common ancestry may predate the emergence of resistance. Previously, Torriani *et al.* (2008) demonstrated that within the European *Z. tritici* population QoI resistance emerged independently on at least four occasions. Given the limited phylogenetic differences observed in the Irish population, it is difficult to determine whether the resistance emerged on multiple occasions or as a single event, from which the different haplotypes subsequently evolved. Amongst the Irish *Z. tritici* isolates screened by Torriani *et al.* (2008), only two haplotypes were identified, of which one was resistant and the other sensitive. Although the number of Irish isolates investigated was relatively low (n = 5), the resistant haplotype was specific to Ireland. Further analysis is required to determine if the current population is related to this haplotype.

Table 4. Analysis of molecular variance (AMOVA) between the three 2017 *Zymoseptoria tritici* populations

Source of variation	df	Sum of squares	Percentage of variation	F_{ST}	P
Among populations	2	2.861	1.05	¼	¼
Within populations	92	98.486	98.95	0.01	0.164

In conclusion, the continued presence of QoI resistance at high frequencies in the Irish population will adversely impact the efficacy of all currently available QoI fungicides, and as such, they are not recommended for the control of *Z. tritici*. Given the high levels of gene flow also detected between the populations, it must be assumed that if resistance to additional fungicides targeting mtDNA encoded proteins to develop, such as the QiIs, they would also spread quite rapidly within the Irish *Z. tritici* population. As such, measures that minimise the potential emergence of such resistances and subsequent selection must be implemented.

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