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Accepted Version

Dooley, H., Shaw, M. W., Mehenni-Ciz, J., Spink, J. and Kildea, S. (2016) Detection of Zymoseptoria tritici SDHI-insensitive field isolates carrying the SdhC-H152R and SdhD-R47W substitutions. Pest Management Science, 72 (12). pp. 2203-2207. ISSN 1526-4998 doi: https://doi.org/10.1002/ps.4269 Available at https://centaur.reading.ac.uk/57491/

To link to this article DOI: http://dx.doi.org/10.1002/ps.4269

Publisher: Wiley

Detection of SDHI insensitivity in a *Zymoseptoria tritici* field population associated with the *SdhC*-H152R and *SdhD*-R47W substitutions

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Abstract

BACKGROUND: Succinate dehydrogenase inhibitor fungicides are important in the management of *Zymoseptoria tritici* in wheat. New active ingredients from this group of fungicides have been introduced recently and are widely used. Because the fungicides act at a single enzyme site, resistance development in *Z. tritici* is classified as medium-to-high risk.

RESULTS: Isolates from Irish experimental plots in 2015 were tested against the SDHI penthiopyrad during routine monitoring. The median of the population was approximately 2 x less sensitive than the median of the baseline population. Two of the 93 isolates were much less sensitive to penthiopyrad than least sensitive of the baseline isolates. These isolates were also insensitive to most of commercially available SDHIs. Analysis of the succinate dehydrogenase coding genes confirmed the presence of the substitutions *SdhC*-H152R and *SdhD*-R47W in the very insensitive isolates.

CONCLUSION: This is the first report showing that the *SdhC*-H152R mutation detected in laboratory mutagenesis studies also exists in the field. The function and relevance of this mutation, combined with *SdhD*-R47W, still needs to be determined.

1 Introduction

Throughout north-western Europe, realising potential winter wheat yields is dependent on the management of diseases, most notably septoria tritici blotch (STB). Caused by the ascomycete pathogen *Zymoseptoria tritici* (synonym *Mycosphaerella graminicola*), STB can reduce yields by up to 50%.[1] At present, control of STB is achieved through the timely application of fungicides. In European *Z. tritici* populations, resistance to the QoI fungicides, [2, 3] in addition to declining azole sensitivity,[4] has developed over the last 10-15 years. Currently, control of STB is heavily dependent on the succinate dehydrogenase inhibitors (SDHIs), azole mixtures, and multi-site acting fungicides such as chlorothalonil and folpet, all of which are used in combination. In addition to the loss of azole sensitivity, the future availability of azoles is in doubt due to EU regulations,[5] so STB control is expected to become increasingly reliant on the SDHI fungicides. Five active ingredients from this group of fungicides are now registered in northern Europe as foliar fungicides for use on cereals; bixafen, boscalid, fluxopyroxad, isopyrazam and penthiopyrad.

SDHIs inhibit fungal respiration by disrupting the functioning of the succinate dehydrogenase (*Sdh*) enzyme within the pathogens' mitochondria, and they provide a broad spectrum of disease control in a wide range of crops including cereals.[6] The specific nature of this control does, however, pose risks for the development of resistance in the target pathogens, and for this reason they are regarded as at a medium-to-high risk of resistance development.[7] To date there are reported to be 12 plant pathogens of economic importance which have developed some level of field resistance to the SDHIs, with resistance induced in an additional two pathogens under laboratory conditions.[8] Field resistance has resulted from mutations in one or more of the *SdhB*, *SdhC* or *SdhD* subunits (*Sdh* enzyme). More than 27 different mutations have been identified across these different pathogens, including alternative amino acids at the same codon position, and depending on the pathogen, mutation and individual active ingredient, resistance factors can vary from low to extremely high.[6]

To gain further insights into potential molecular mechanisms of resistance to the SDHIs in *Z. tritici*, laboratory induced resistant mutants have been analysed by several groups.[9-12] In those studies mutations in one or all of the three subunits were found and the most commonly identified mutations included *SdhB*-H267Y, *SdhC*-A84V and *SdhC*-H152R. However, the effects of the different mutations on sensitivity depended on the SDHI used and, in some instances, on the genetic background of the *Z. tritici* isolates tested. Mutations at some of the codons identified in the mutagenesis studies have been identified in field strains (*SdhB*-N225T, *SdhC*-T79N, and *SdhC*-N86S) in different locations throughout north-western Europe, although resistance factors have been reported to be low.[8]

In 2015, during monitoring of a *Z. tritici* field population from an experimental trial against the SDHI penthiopyrad, isolates exhibiting decreased SDHI sensitivity were discovered. A selection of isolates from that population was further examined and compared to a larger collection, representing populations prior to the recent commercialisation of the 3rd generation SDHI fungicides, to confirm their sensitivity and potential cause of insensitivity.

2 Materials & Methods

2.1 Origin of strains

Winter wheat plots of the variety Cordiale, treated with the SDHI penthiopyrad, the azole prothioconazole or the multi-site folpet, were randomly sampled for *Z. tritici* infected leaves. From these, 93 strains were isolated and their sensitivity to the SDHI fungicide penthiopyrad determined as described by Dooley et al.[13] Eight of these isolates were selected from that collection based on sensitivity to penthiopyrad (Figure 1). Four were highly sensitive with EC_{50} values within the baseline range (EC_{50} values < 1.6 mg/l), two had moderate sensitivity (EC_{50} values slightly above the highest EC_{50} in the baseline range, > 1.6 mg/l) and two had low sensitivity (EC_{50} values > 30 mg/l).

The baseline sensitivity was based on a collection of 209 field isolates from the years 2005-2010. Sample sizes were: 2005, n = 26; 2006, n = 36; 2007, n = 19; 2009, n = 80; 2010, n = 48. Isolates came from commercial fields, representing 21 locations in Ireland, and four locations in the UK for comparison. The UK isolates (courtesy of J. Blake, ADAS) were collected in 2010 only.

2.2 Fungicide sensitivity

Sensitivity of the whole 2015 collection was determined to penthiopyrad initially, using a microtitre plate assay as described by Dooley et al.[13] Sensitivity of eight selected strains representing the range of sensitivities present was determined to boscalid, bixafen, fluopyram, fluxapyroxad, isopyrazam, and penthiopyrad using the same assay used for the initial screen but with a greater range of test concentrations: from 0 to 100 mg/l with 12 dilutions, and plates were replicated three times. The sensitivity of all baseline isolates was determined to the same six SDHIs mentioned above using the same assay with appropriate concentration adjustments. Following incubation, sensitivity of each isolate was determined by assessing fungal growth, measured as light absorbance at 405 nm using Synergy-HT plate reader and Gen5™ microplate software (BioTek Instruments, Inc., USA) and subsequently expressed as the fungicide concentration inhibiting growth by 50% (EC_{50}) by fitting a logistic curve to percentage inhibition data using XLfit (IDBS Inc., UK). Standard error was calculated for the EC_{50} values of the eight individual isolates.

All statistical analyses were carried out using GenStat V 14.1.0. For the baseline collection the Shapiro-Wilk test was used to test for normality. A randomisation test was used to estimate the probability that the two most insensitive isolates found would be found in the 2015 sample if there were actually a constant frequency in all samples.

2.3 Sequence analysis of *SdhB*, *SdhC*, and *SdhD* subunits

The DNA sequences of the eight isolates from 2015 and a subset of 96 isolates (46% of the baseline collection) from the baseline collection were determined. Baseline isolates were chosen based on their sensitivity (EC_{50} value) to isopyrazam. From each of the five years 19 strains were chosen, six of which had low, seven had medium and six had high Isopyrazam EC_{50} values relative to that year's collection. DNA extraction, PCR amplification, sequencing of each subunit and analysis was performed as previously described by Dooley et al.[13] with the exception that both forward and reverse primers were used to sequence the 2015 isolates.

3 Results

3.1 Sensitivity of isolates

A wide range of sensitivities to the SDHI fungicide penthiopyrad (between 0.02 and > 30 mg/l) was observed amongst the 93 field isolates from 2015 (Figure 1). The median sensitivity of the 2015 collection shifted towards EC_{50} values about 50% greater than the baseline, from EC_{50} values of 0.163 mg/l to 0.26 mg/l, and the distribution was bi-modal (Figure 1). The sensitivity to the other SDHIs of the eight isolates tested further was, with the exception of fluopyram, consistent with the response to penthiopyrad (Table 1). Isolates initially selected as highly sensitive to penthiopyrad had sensitivities similar to the mean/median baseline sensitivity of the other SDHIs, again with the exception of fluopyram (see Table 2 for baseline sensitivity). Those with moderate sensitivity were individually within the baseline normal or skewed normal distributions (see Table 2 for baseline sensitivity). The two isolates initially selected as having very low sensitivity to penthiopyrad had high resistance factors (Table 1) and did not lie in the original normal distributions, where applicable ($P < 0.001$ for EC_{50} to all fungicides except fluopyram on the null hypothesis of a normal distribution). Although both less sensitive isolates were found in 2015, there is no convincing evidence for any increase in frequency in the field since the start of commercial use of SDHI fungicides ($P = 0.094$ by direct calculation or randomisation test). However, the 2015 sample distribution as a whole is less sensitive than the baseline (Kolmogorov 2-sample test, $D = 0.31$, $P < 0.001$)

3.2 Variation in the *Sdh* subunits of isolates

In the eight isolates from 2015 only a single synonymous substitution was observed in the *SdhB* subunit. A large number of variations were observed in the *SdhC* subunit, however only five of these resulted in changes in the target protein (Table 1). The amino acid substitutions *SdhC*-R13P, *SdhC*-N33T and *SdhC*-N34T had no observable impact on SDHI sensitivity as measured; *SdhC*-N33T and *SdhC*-N34T were detected widely within the baseline collection (Table 3); *SdhC*-N79T was detected in a single strain and associated with medium levels of SDHI sensitivity. The isolates OP15.13 and OP15.15, both displaying high resistance factors towards penthiopyrad and other SDHIs, contained the *SdhC*-H152R and *SdhD*-R47W substitutions.

4 Discussion

The six 2015 isolates selected as moderately sensitive or highly sensitive were similar to baseline isolates in both sensitivity and mutation spectrum. The *SdhC*-N79T mutation was present in one of these moderately sensitive isolates, confirming the low resistance factors previously reported by FRAC.[8] The two isolates which had the mutations *SdhC*-H152R and *SdhD*-R47W were extremely insensitive to all SDHIs, with the exception of fluopyram. This cross-resistance amongst SDHIs was also seen in the baseline data (data not shown) and is in agreement with Fraaije et al. [11] who found clear positive correlations between different SDHIs, and Schürch and Cordette [14] who found similar patterns but with weaker relationships. The *SdhC*-H152R mutation has previously been identified by both Stammler et al. [10] and Scalliet et al. [12] in mutagenesis studies; the latter reporting high resistance factors to the majority of newer SDHIs. The incomplete cross-resistance between fluopyram and the other SDHIs, which was observed in the baseline data (data not shown), was also demonstrated by Scalliet et al,[12] who found that an isolate with the *SdhC*-H152R mutation which grew in the presence of boscalid and isopyrazam, was all but restricted in the presence of fluopyram. This incomplete cross-resistance has also been demonstrated in other pathogens such as *Alternaria alternate*,[15] *A. solani*,[16] *Botrytis cinerea*,[17] and *Corynespora cassicola*[18].

This is the first finding of *SdhC*-H152R in a *Z. tritici* field population, and as such represents an important development. Whilst Scalliet et al. [12] demonstrated the mutation *SdhC*-H152R did not affect the ability of *Z. tritici* laboratory mutated strains to infect and cause disease, they did report a reduction in enzyme activity. As our isolates were retrieved from the field at a frequency (0.66%, 95% CI 0.08%-1.8%) much larger than the mutation rate (typically less than 10^{-9} for point mutations), they must be able to infect and cause disease. Whether they suffer a fitness penalty, and what role if any the mutation *SdhD*-R47W plays, remains to be determined.

Irrespective of potential fitness penalties it must be assumed that the continued widespread use of SDHIs is likely to result in an increase in frequency of the alleles associated with high resistance factors because of the very strong selection imposed by good current control levels.[19] Such an increase will adversely affect the efficacy of those SDHIs currently available as foliar applied products for STB control. Currently the SDHIs are an essential tool in the control of STB in in north-western Europe. It is imperative that all available measures are taken to maintain their excellent field performance against *Z. tritici* for as long as possible. Continued monitoring of *Z. tritici* field populations is essential to be able to identify changes in sensitivity and mutations which cause those changes. Fungicide resistance management strategies, such as reductions in the number of applications of active ingredients from a single group and mixing with effective fungicide partners,[20] particularly multi-site acting fungicides, must be used to help slow the selection of resistant strains. Disease incidence should be reduced by using host resistance[21, 22] and any proven agronomic practices which reduce *Z. tritici* population growth rates during the period of application of fungicide implemented, since they will reduce the rate of selection.[23-25]

Acknowledgements

The authors would like to thank Liz Glynn and Jim Grace from Teagasc, and student Aurélien Lepennetier for their help with laboratory testing. This research has been funded by Teagasc under the Walsh Fellowship scheme and the Irish Department of Agriculture, Food and Marine under their research stimulus fund, grant number 11S113.

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Figure 1 Frequency distribution of baseline isolates (n = 209) and 2015 isolates (n = 93) to the SDHI fungicide penthiopyrad. Re-tested isolates for which *Sdh* sequence was obtained are marked by arrows.