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

4
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13 **Abstract**

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

18 RESULTS: Isolates from Irish experimental plots in 2015 were tested against the SDHI
19 penthiopyrad during routine monitoring. The median of the population was approximately 2
20 x less sensitive than the median of the baseline population. Two of the 93 isolates were much

1 less sensitive to penthiopyrad than least sensitive of the baseline isolates. These isolates were
2 also insensitive to most of commercially available SDHIs. Analysis of the succinate
3 dehydrogenase coding genes confirmed the presence of the substitutions *SdhC*-H152R and
4 *SdhD*-R47W in the very insensitive isolates.

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

8 **1 Introduction**

9 Throughout north-western Europe, realising potential winter wheat yields is dependent on the
10 management of diseases, most notably septoria tritici blotch (STB). Caused by the
11 ascomycete pathogen *Zymoseptoria tritici* (synonym *Mycosphaerella graminicola*), STB can
12 reduce yields by up to 50%.¹ At present, control of STB is achieved through the timely
13 application of fungicides. In European *Z. tritici* populations, resistance to the QoI fungicides,
14 ^{2, 3} in addition to declining azole sensitivity,⁴ has developed over the last 10-15 years.
15 Currently, control of STB is heavily dependent on the succinate dehydrogenase inhibitors
16 (SDHIs), azole mixtures, and multi-site acting fungicides such as chlorothalonil and folpet,
17 all of which are used in combination. In addition to the loss of azole sensitivity, the future
18 availability of azoles is in doubt due to EU regulations,⁵ so STB control is expected to
19 become increasingly reliant on the SDHI fungicides. Five active ingredients from this group
20 of fungicides are now registered in northern Europe as foliar fungicides for use on cereals;
21 bixafen, boscalid, fluxopyroxad, isopyrazam and penthiopyrad.

22 SDHIs inhibit fungal respiration by disrupting the functioning of the succinate
23 dehydrogenase (*Sdh*) enzyme within the pathogens' mitochondria, and they provide a broad

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

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

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

1 **2 Materials & Methods**

2 **2.1 Origin of strains**

3 Winter wheat plots of the variety Cordiale, treated with the SDHI penthiopyrad, the azole
4 prothioconazole or the multi-site folpet, were randomly sampled for *Z. tritici* infected leaves.
5 From these, 93 strains were isolated and their sensitivity to the SDHI fungicide penthiopyrad
6 determined as described by Dooley et al.¹³ Eight of these isolates were selected from that
7 collection based on sensitivity to penthiopyrad (Figure 1). Four were highly sensitive with
8 EC₅₀ values within the baseline range (EC₅₀ values < 1.6 mg/l), two had moderate sensitivity
9 (EC₅₀ values slightly above the highest EC₅₀ in the baseline range, > 1.6 mg/l) and two had
10 low sensitivity (EC₅₀ values > 30 mg/l).

11 The baseline sensitivity was based on a collection of 209 field isolates from the years
12 2005-2010. Sample sizes were: 2005, n = 26; 2006, n = 36; 2007, n = 19; 2009, n = 80;
13 2010, n = 48. Isolates came from commercial fields, representing 21 locations in Ireland, and
14 four locations in the UK for comparison. The UK isolates (courtesy of J. Blake, ADAS) were
15 collected in 2010 only.

16 **2.2 Fungicide sensitivity**

17 Sensitivity of the whole 2015 collection was determined to penthiopyrad initially, using a
18 microtitre plate assay as described by Dooley et al.¹³ Sensitivity of eight selected strains
19 representing the range of sensitivities present was determined to boscalid, bixafen,
20 fluopyram, fluxapyroxad, isopyrazam, and penthiopyrad using the same assay used for the
21 initial screen but with a greater range of test concentrations: from 0 to 100 mg/l with 12
22 dilutions, and plates were replicated three times. The sensitivity of all baseline isolates was
23 determined to the same six SDHIs mentioned above using the same assay with appropriate

1 concentration adjustments. Following incubation, sensitivity of each isolate was determined
2 by assessing fungal growth, measured as light absorbance at 405 nm using Synergy-HT plate
3 reader and Gen5™ microplate software (BioTek Instruments, Inc., USA) and subsequently
4 expressed as the fungicide concentration inhibiting growth by 50% (EC₅₀) by fitting a logistic
5 curve to percentage inhibition data using XLfit (IDBS Inc., UK). Standard error was
6 calculated for the EC₅₀ values of the eight individual isolates.

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

11 **2.3 Sequence analysis of *SdhB*, *SdhC*, and *SdhD* subunits**

12 The DNA sequences of the eight isolates from 2015 and a subset of 96 isolates (46% of the
13 baseline collection) from the baseline collection were determined. Baseline isolates were
14 chosen based on their sensitivity (EC₅₀ value) to isopyrazam. From each of the five years 19
15 strains were chosen, six of which had low, seven had medium and six had high Isopyrazam
16 EC₅₀ values relative to that year's collection. DNA extraction, PCR amplification,
17 sequencing of each subunit and analysis was performed as previously described by Dooley et
18 al.¹³ with the exception that both forward and reverse primers were used to sequence the 2015
19 isolates.

1 **3 Results**

2 **3.1 Sensitivity of isolates**

3 A wide range of sensitivities to the SDHI fungicide penthiopyrad (between 0.02 and > 30
4 mg/l) was observed amongst the 93 field isolates from 2015 (Figure 1). The median
5 sensitivity of the 2015 collection shifted towards EC₅₀ values about 50% greater than the
6 baseline, from EC₅₀ values of 0.163 mg/l to 0.26 mg/l, and the distribution was bi-modal
7 (Figure 1). The sensitivity to the other SDHIs of the eight isolates tested further was, with
8 the exception of fluopyram, consistent with the response to penthiopyrad (Table 1). Isolates
9 initially selected as highly sensitive to penthiopyrad had sensitivities similar to the
10 mean/median baseline sensitivity of the other SDHIs, again with the exception of fluopyram
11 (see Table 2 for baseline sensitivity). Those with moderate sensitivity were individually
12 within the baseline normal or skewed normal distributions (see Table 2 for baseline
13 sensitivity). The two isolates initially selected as having very low sensitivity to penthiopyrad
14 had high resistance factors (Table 1) and did not lie in the original normal distributions,
15 where applicable ($P < 0.001$ for EC₅₀ to all fungicides except fluopyram on the null
16 hypothesis of a normal distribution). Although both less sensitive isolates were found in
17 2015, there is no convincing evidence for any increase in frequency in the field since the start
18 of commercial use of SDHI fungicides ($P = 0.094$ by direct calculation or randomisation
19 test). However, the 2015 sample distribution as a whole is less sensitive than the baseline
20 (Kolmogorov 2-sample test, $D = 0.31$, $P < 0.001$)

21 **3.2 Variation in the *Sdh* subunits of isolates**

22 In the eight isolates from 2015 only a single synonymous substitution was observed in the
23 *SdhB* subunit. A large number of variations were observed in the *SdhC* subunit, however only

1 five of these resulted in changes in the target protein (Table 1). The amino acid substitutions
2 *SdhC*-R13P, *SdhC*-N33T and *SdhC*-N34T had no observable impact on SDHI sensitivity as
3 measured; *SdhC*-N33T and *SdhC*-N34T were detected widely within the baseline collection
4 (Table 3); *SdhC*-N79T was detected in a single strain and associated with medium levels of
5 SDHI sensitivity. The isolates OP15.13 and OP15.15, both displaying high resistance factors
6 towards penthiopyrad and other SDHIs, contained the *SdhC*-H152R and *SdhD*-R47W
7 substitutions.

8 **4 Discussion**

9 The six 2015 isolates selected as moderately sensitive or highly sensitive were similar to
10 baseline isolates in both sensitivity and mutation spectrum. The *SdhC*-N79T mutation was
11 present in one of these moderately sensitive isolates, confirming the low resistance factors
12 previously reported by FRAC.⁸ The two isolates which had the mutations *SdhC*-H152R and
13 *SdhD*-R47W were extremely insensitive to all SDHIs, with the exception of fluopyram. This
14 cross-resistance amongst SDHIs was also seen in the baseline data (data not shown) and is in
15 agreement with Fraaije et al.¹¹ who found clear positive correlations between different
16 SDHIs, and Schürch and Cordette¹⁴ who found similar patterns but with weaker
17 relationships. The *SdhC*-H152R mutation has previously been identified by both Stammler et
18 al.¹⁰ and Scalliet et al.¹² in mutagenesis studies; the latter reporting high resistance factors to
19 the majority of newer SDHIs. The incomplete cross-resistance between fluopyram and the
20 other SDHIs, which was observed in the baseline data (data not shown), was also
21 demonstrated by Scalliet et al.,¹² who found that an isolate with the *SdhC*-H152R mutation
22 which grew in the presence of boscalid and isopyrazam, was all but restricted in the presence
23 of fluopyram. This incomplete cross-resistance has also been demonstrated in other

1 pathogens such as *Alternaria alternata*,¹⁵ *A. solani*,¹⁶ *Botrytis cinerea*,¹⁷ and *Corynespora*
2 *cassicola*¹⁸.

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

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

1 practices which reduce *Z. tritici* population growth rates during the period of application of
2 fungicide implemented, since they will reduce the rate of selection.²³⁻²⁵

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8

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39

1

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