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Cross-resistance between myclobutanil and tebuconazole and the genetic basis of tebuconazole resistance in *Venturia inaequalis*

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Abstract

BACKGROUND: Myclobutanil is one of the most widely used demethylation inhibitor (DMI) fungicides for the management of apple scab, caused by *Venturia inaequalis*. Strains of *V. inaequalis* resistant to myclobutanil have been reported across the world. Tebuconazole, another DMI fungicide, has been proposed as an alternative to myclobutanil, and the extent of cross-resistance with myclobutanil therefore needs to be evaluated. The sensitivity to tebuconazole and myclobutanil of a total of 40 isolates was determined. Half the isolates came from an isolated orchard which had never been sprayed with fungicides and half from orchards sprayed regularly with myclobutanil, but still with disease control problems. The progeny of a tebuconazole resistant (R) × sensitive (S) *V. inaequalis* cross were analyzed in order to improve understanding of the genetic control of tebuconazole sensitivity.

RESULTS: There is cross-resistance between myclobutanil and tebuconazole ($r = 0.91$; $P < 0.001$). Sensitivity to tebuconazole of the progeny of a R × S cross varied quantitatively in a pattern which implied at least two gene loci differing between the parental strains. In addition, the asymmetric distribution of the sensitivity in the progeny implied possible epistatic effects.

CONCLUSION: Resistance to myclobutanil and tebuconazole is strongly correlated. At least two genes are involved in the control of tebuconazole resistance in *V. inaequalis*.
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Keywords: myclobutanil; tebuconazole; cross-resistance; apple scab; *Venturia inaequalis*; quantitative genetics

1 INTRODUCTION

Apple scab is a disease of apple trees caused by the ascomycete fungus *Venturia inaequalis*. As the most important disease affecting the apple crop in the UK, apple scab is controlled through a range of methods, with heavy reliance on fungicides. Among these fungicides, the demethylation inhibitor (DMI) family has been one of the most widely used for the control of apple scab.[1] Myclobutanil is one of the members of the DMI family for which an increase in resistance in *V. inaequalis* has been reported.[2-7] As there are only a limited number of products available for control of apple scab, the spread of fungicide resistance could leave growers without a sufficient number of products for scab control.[4]

The mechanisms involved in DMI fungicide resistance in *V. inaequalis* are not entirely known. Candidates that could play a role in resistance development include transport proteins and changes in the expression or structure of the target demethylase protein.[2,6] In apple scab, laboratory mutants have confirmed the involvement of transport systems in fungicide efflux.[8,9] Studies in field isolates have found correlation of fungicide resistance with overexpression of the *CYP51A1* gene,[10,11] although not all field isolates with resistance displayed this mechanism,[10] suggesting the existence of other mechanisms.

In other species resistance mechanisms to DMI fungicides vary. For example, in *Zymoseptoria tritici*, although most attention has focused on changes in the target *CYP51* gene,[12] several studies support a quantitative resistance pattern. For instance, resistance to the DMI propiconazole is controlled by multiple genes[13] and Mavroeidi[14] found a continuous and transgressive distribution of sensitivity to the DMI fluquinconazole in the progeny of a cross, with evidence for epistasis between loci involved. In other species, crosses suggest a variety of genetic architectures leading to resistance. For example in the case of *Tapesia yallundae*, resistance to the DMI prochloraz has a major single gene component,

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with only a minor contribution by other genes in some of the isolates with higher resistance.[15] Carter *et al.*[16] found in some resistant isolates of *Pyrenopeziza brassicae* a gene other than *CYP51* with a major effect.

A previous study[17] in *V. inaequalis* assumed a single-gene form of control of azole resistance. Stanis *et al.*[17] observed a 1:1 pattern in nine crosses involving resistant–sensitive crosses, resistant–resistant crosses and sensitive–sensitive crosses. However, only one discriminatory dose was used, and isolates were considered resistant or sensitive depending on whether they were able to grow at this particular discriminatory dose.[17] The results from the use of a single discriminatory dose when the distribution of sensitivity values in the field is not bimodal[1,2] depend strongly on the choice of this particular dose, and hence may not be able to reveal control by multiple loci.

Although the target site of DMI fungicides is, by definition, the demethylation step in sterol biosynthesis,[6] cross-resistance between DMI fungicides is not consistent between pathogens and sometimes within pathogens, varying with factors such as the origins of fungal isolates.[1] As such, it is not usually advisable to make inferences about cross-resistance in a particular pathogen based on inference from other pathogens.[1] In apple scab, DMI cross-resistance has been reported between several fungicide pairs, including myclobutanil-flusilazole, myclobutanil-fenarimol and myclobutanil-fenbuconazole.[2,6,18]

The current study was designed primarily to evaluate the suitability of tebuconazole as an alternative to myclobutanil for the management of apple scab. To this end, the tebuconazole sensitivity in a baseline *V. inaequalis* population and its cross-resistance with myclobutanil was studied. During the evaluation, some isolates were shown to have relatively high resistance to tebuconazole. To improve understanding of how this resistance may further evolve, the distribution of ED_{50} in the offspring of a cross between tebuconazole resistant and sensitive isolates was used to test some simple hypotheses about the genetic control of the differences between the parents. This is particularly simple because the parents and offspring are both haploid. We considered the following hypotheses: (H1) alleles at a single locus control the bulk of the differences; (H2) alleles at two or more loci control the bulk of the differences; and (H3) alleles at two or more loci control the bulk of the differences but do not interact epistatically.

2 MATERIALS AND METHODS

2.1 Collection of field isolates

Scab isolates were classified as baseline (20 isolates) or candidate resistant isolates (20 isolates). The baseline isolates came from trees from a noncommercial orchard in the west of England (Worcestershire) that has never been treated with fungicides.[1,19] The samples from this orchard were all collected in early spring 2005 (hence more likely to originate from ascospore infections) and stored at −20 °C as leaf discs. Sixteen of the 20 candidate resistant isolates were provided by consultants who collected one scabbed leaf per tree from four commercial orchards in Kent (the south east of England) with known problems of scab control in 2009. One, two, three and ten isolates were obtained from the four commercial orchards, respectively. Candidate resistant isolates came from orchards which typically received between 12 and 15 sprays annually, including myclobutanil. The remaining four candidate resistant isolates were collected in 2015 at NIAB EMR (Kent) from potted trees on which severe scab developed despite a number of applications of myclobutanil.

Single-spore isolates were obtained from leaf discs with scab lesions. To isolate conidia, leaf discs were placed in a microcentrifuge tube with deionized water (1 mL) and conidia were released into the water by crushing with a pestle fitting the tube, followed by a few seconds of vortex mixing. A short spin in a centrifuge was used to make the leaf material settle in the bottom of the tube. For the commercial orchard lesions, 16 batches of one leaf disc were prepared this way; for the baseline isolates, three batches each of about ten leaf discs taken randomly across the orchard were used; and for the candidate resistant isolates from the NIAB EMR site, a similar approach to the baseline isolates was used, but with separate batches depending on the tree variety where the lesion came from. Conidia in the resulting supernatant were counted on a haemocytometer, and concentrations adjusted to 8×10^4 conidia mL^{-1}. Two dilutions were prepared out of the conidia suspension, one by a factor of ten and another by a factor of 20; and the undiluted suspension and the two dilutions were plated with a glass spreader on plain water agar (PWA) prepared with Agar technical No. 3 (Oxoid UK, Basingstoke, Hampshire, UK; 1.2% w/v) amended with rifamycin (Sigma-Aldrich UK, Gillingham, Dorset, UK; 0.2 ppm). Plates were incubated at 20 °C overnight.

After incubation, individual germinating spores were picked with a needle under a dissecting microscope from areas of the plate where spores were abundant but well-spaced, and transferred to PDA plates (Oxoid UK; 39 g L^{-1}) amended with rifamycin (Sigma-Aldrich; 0.2 ppm).

2.2 Crossing of isolates and selection of spores

From the 40 field isolates, 36 crosses between very sensitive isolates to tebuconazole (all from the baseline orchard and with $lnED_{50}$ values that ranged from 0.12 mg L^{-1} to 0.85 mg L^{-1}) and very resistant isolates (all from the candidate resistant group and with $lnED_{50}$ values that ranged from 3.03 to 18.3 mg L^{-1}) were attempted following the protocol of Barbara *et al.*[20] Briefly, 4-mm mycelial plugs were placed 2 cm apart on Apple Leaf Malt Extract Agar[21] prepared using senescent apple leaves collected on 10 November (2015). Plates were kept at ≈20 °C for five weeks, then at 4 °C for six months after which single ascospores were isolated.

Among the crosses with adequate numbers of offspring, the cross with the maximum difference in ED_{50} to tebuconazole between sensitive and resistant parents, AF28 (ED_{50} = 0.12 mg L^{-1}) × Spartan 1 (ED_{50} = 3.36 mg L^{-1}), was chosen for further analysis. From this cross, 200 germinating ascospores with a sufficient distance from other spores were picked and placed on PDA plates (Oxoid; 39 g L^{-1}) amended with rifamycin (Sigma-Aldrich; 0.2 ppm), five spores on each individual plate.

2.3 Fungicide tests

The field isolates were tested for fungicide sensitivity to both myclobutanil (Systhane 20EW, Dow AgroSciences, Indianapolis, IN, USA) and tebuconazole (Folicur 25 EW, Bayer Crop Science UK, Cambridge, UK). The cross progeny were tested with tebuconazole only.

For each field isolate, its level of sensitivity was tested by subculturing on PDA media (Oxoid) amended with the following concentrations for each of the two fungicides separately (myclobutanil and tebuconazole): 4, 1, 0.1, 0.01 and 0 mg L^{-1}. This concentration range was selected by taking into account discriminatory doses for myclobutanil used in previous studies.[6,22,23,24] For each combination of isolate and concentration, there was

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one plate with three to four mycelial plugs (4 mm), depending on the quantity of material available for that isolate at the time of experimentation. In a few cases, owing to the lack of fungal material, only two plugs were used. After three weeks, two perpendicular diameters of the resulting culture from each plug were measured. The whole experiment was repeated once over time.

On each day when subcultures were made, the same number of isolates from both baseline and candidate resistant categories were used, to minimize the effect of batch variability on comparisons between the two groups of isolates.

A total of 150 ascospores from the AF28 × Spartan1 cross were initially maintained in PDA media (Oxoid) amended with rifamycin (Sigma-Aldrich; 0.2 ppm). The ED_{50} to tebuconazole of 81 of these cultures was measured, after excluding contaminated isolates. Because *V. inaequalis* grows very slowly in culture, any contamination during the growth assay is very obvious and unlikely to affect the estimates of ED_{50}. Parental isolates were tested alongside the progeny. Four tebuconazole concentrations (4, 1, 0.1 and 0.01 mg L^{-1}) and a control without the fungicide were used. For each concentration, there were two plates, each with two plugs. After three weeks, two perpendicular diameters of the resulting culture from each plug were measured.

The plate was treated as a biological replicate in both the data analyses of the field isolates and the progeny, with the mean diameters (mean of the two perpendicular diameter measurements) of all the plugs on one plate averaged for further analysis.

2.4 Data handling

GENSTAT v16[25] was used to analyze all of the data.

The same approach was used to obtain $\ln ED_{50}$ values, where ln indicates natural-logarithm, of the field isolates and the progeny of the AF28 × Spartan1 cross. For each isolate, its $\ln ED_{50}$ was estimated through a logistic model relating the mean diameter of all colonies within a plate to concentrations (ln-transformed). To allow the inclusion of the control in the analyses, its concentration was replaced before transformation by a figure two orders of magnitude (0.0001 mg L^{-1}) below that of the lowest fungicide concentration used in the test (0.01 mg L^{-1}). With only five concentrations, a very parsimonious function needs to be used to provide estimates of $\ln ED_{50}$. The $\ln ED_{50}$ estimates were taken as the parameter *M* in the general logistic equation [Eqn (1)], assuming no growth at infinite dose:

$$y = \frac{C}{1 + e^{((-B)*(x-M))}} \quad (1)$$

where *x* is the natural logarithm of fungicide concentration, *y* is the growth measurement and *C* is the growth at the zero dose. This was reduced to a two-parameter function, providing 3 degrees of freedom for residual errors, by fixing the slope parameter (*B*) at an intermediate value chosen to give estimates of other parameters which were insensitive to the exact slope. Fit was checked visually. Resistance ratios of individual isolates were calculated relative to the mean $\ln ED_{50}$ of the baseline isolates and then back-transformed.

In order to study cross-resistance, the log-transformed values of ED_{50} ($\ln ED_{50}$) for both chemicals were plotted against each other. The confidence ellipses for each isolate were calculated from the estimated uncertainty in the $\ln ED_{50}$. Spearman rank correlation (ρ) was calculated to reduce the influence of strains with extreme low or high $\ln ED_{50}$ values in some isolates. Pearson correlations also were obtained for the baseline isolates and the candidate resistant isolates, with calculations done for the baseline and candidate resistant datasets combined and separately.

Eight isolates in the candidate resistant group grew in concentrations of myclobutanil so high that the logistic model fitting failed to estimate their $\ln ED_{50}$. To use them in the cross-resistance calculations, their $\ln ED_{50}$ was estimated visually from the shape of the growth curve. It is more likely that the $\ln ED_{50}$ values of these isolates were underestimated rather than overestimated, so their upper error bar was taken as the largest standard error (SE) estimated in all the other isolates and the lower error bars were calculated from the mean of the SEs of all other isolates.

2.5 Genetic architecture of the resistance

The three hypotheses stated in the introduction were tested by comparing the observed distribution of $\ln ED_{50}$ in the offspring of the cross with the distribution expected under each of the three hypotheses. Derivations of these expected distributions are provided in the supplementary information. To test the hypotheses, the observed distribution was compared with each of the predicted distributions using Kolmogorov–Smirnov 1-sample tests.

3 RESULTS

3.1 Characteristics of the populations

In the baseline orchard, sensitivity (measured as ED_{50}) to myclobutanil ranged from 0.14 mg L^{-1} to 47 mg L^{-1} [mean ED_{50} = 2.3 mg L^{-1}, confidence interval (CI) 1.2–4.5 mg L^{-1}, back-transformed from the ln scale, CV = 183%]. In the candidate resistant group, eight isolates were too resistant for the model fitting to determine the inflection point of the curve. For the remaining isolates, the sensitivity ranged from 4.3 to 72.4 mg L^{-1}, and the resistance ratios relative to the mean of the baseline from 1.8 to 31. In the baseline orchard, sensitivity to tebuconazole ranged from 0.02 to 3.0 mg L^{-1} (mean ED_{50} = 0.6 mg L^{-1}, CI 0.34–1.1 mg L^{-1}, back-transformed from the ln scale, CV = 594%). In the candidate resistant isolates, sensitivity to tebuconazole varied from 1.7 to 25 mg L^{-1} with resistance ratios ranging from 2.8 to 41.2. The $\ln ED_{50}$ values of the baseline and candidate resistant isolates differed (Wilcoxon signed rank test $P < 0.001$) for both myclobutanil and tebuconazole.

3.2 Baseline sensitivity distribution

The distribution of the baseline data was indistinguishable from a normal distribution for both myclobutanil and tebuconazole (Kolmogorov–Smirnov $P = 0.98$; Shapiro–Wilks $P = 0.99$; Supporting Information, Fig. S1). Eight candidate resistant isolates had very high ED_{50}; the distribution of the candidate resistant isolates was, therefore, not normally distributed.

3.3 Cross-resistance

There was a strong correlation (Fig. 1) between the $\ln ED_{50}$ values of the two chemicals (With r denoting Pearson correlation and ρ Spearman rank, $r = 0.91$, $P < 0.001$; $\rho = 0.89$, $P < 0.001$). This correlation was stronger in the baseline population ($r = 0.86$, $P < 0.001$) than in the candidate resistant population ($r = 0.64$, $P = 0.002$). It is important to note that the wide CIs for some of the resistant isolates automatically reduced the correlation coefficient.

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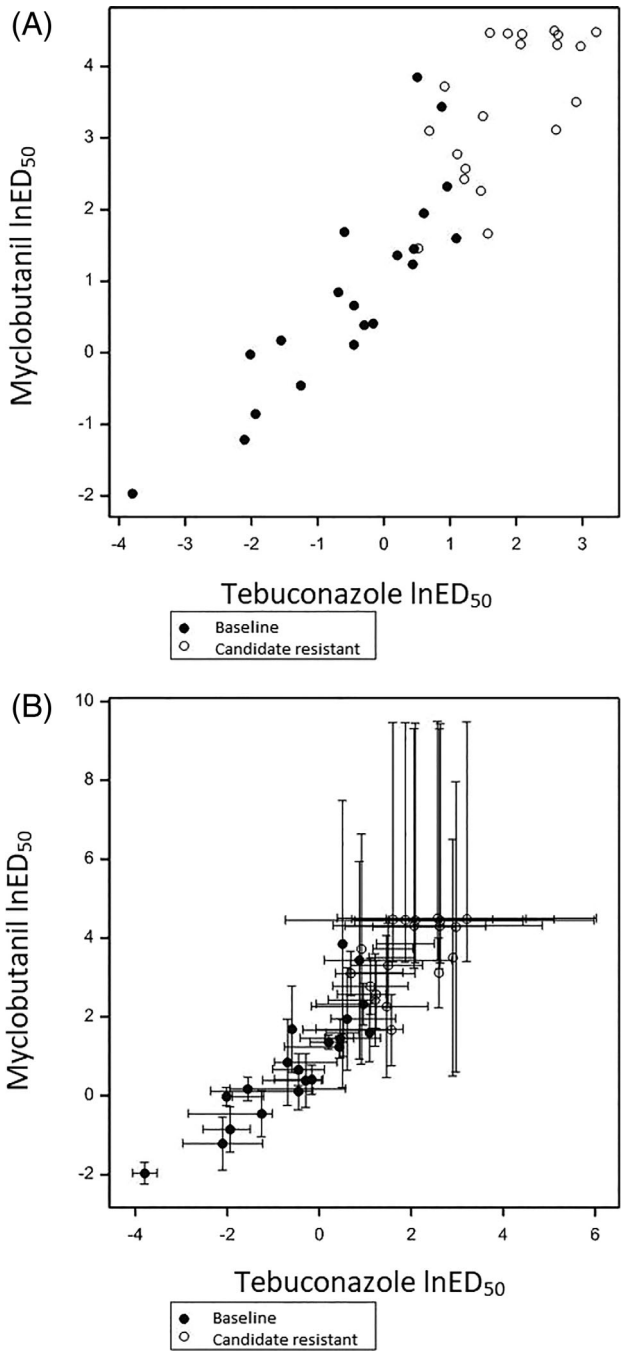

Figure 1. (A) Cross-resistance between myclobutanil and tebuconazole in isolates from one baseline orchard never directly exposed to fungicide and several candidate resistant orchards. (B) gives the same data with error bars showing 2 × SE of each estimate on a ln scale. Eight isolates grew in concentrations of myclobutanil so high that the logistic model fitting failed. These isolates have been included in the graph with ED_{50} values for myclobutanil roughly estimated from the shape of their growth scatter plots, an upper error corresponding to the highest SE in other isolates, and a lower error corresponding to the mean of the SEs of the remaining isolates.

3.4 Distribution of the sensitivity of the progeny of a resistant-sensitive cross to DMI fungicide tebuconazole

The cumulative distribution of the sensitivity values of the cultures in the progeny of the AF28 × Spartan 1 cross, including the two parental isolates (Fig. 2), was continuous with no apparent

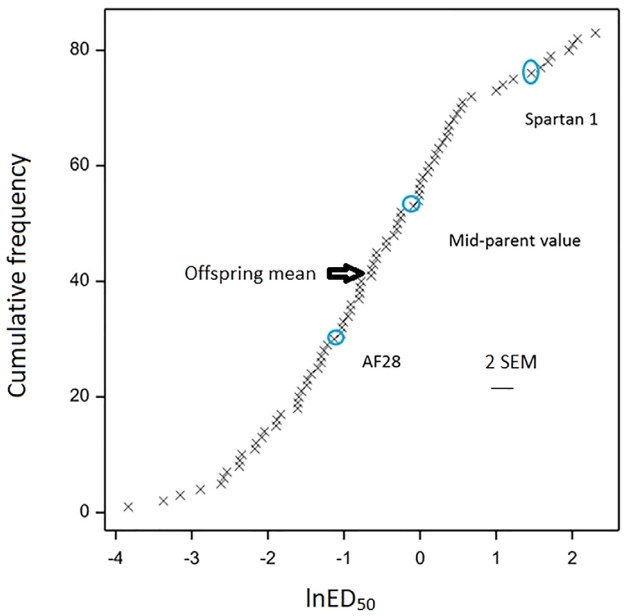

Figure 2. Cumulative distribution of the sensitivity levels of the AF28 × Spartan 1 progeny. The sensitivity levels of the sensitive parent, AF28, the resistant parent, Spartan 1, tested in the same test series as the progeny, and the mid-point of sensitivity between the parents have been marked with a circle. The mean $lnED_{50}$ of the progeny is indicated by an arrow. SEM, SE of the mean.

discontinuities allowing a division into distinct classes. This cumulative distribution of the ascospore progeny did not resemble that expected from a single segregating locus (Fig. 3; one-sample

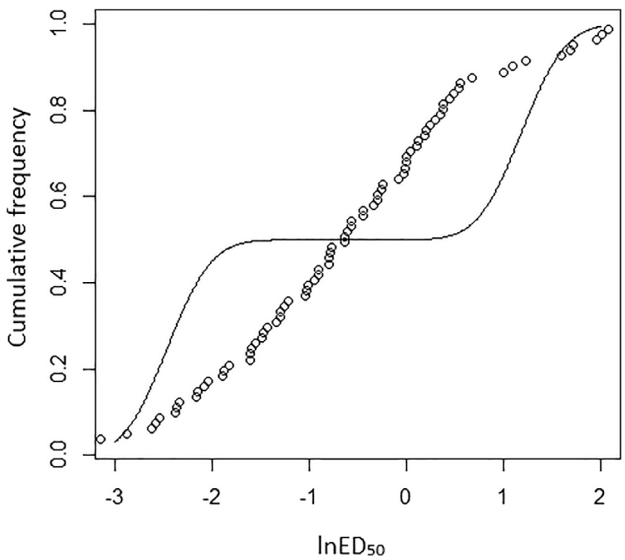

Figure 3. Comparison between the observed cumulative distribution of the $lnED_{50}$ values of the progeny of the AF28 × Spartan 1 cross (data points) and the expected distribution under the hypothesis that the difference between the parental isolates is due to one locus (continuous line). The sensitivity range and number of ascospores in the expected distribution are the same as in the observed data. A few of the most extreme values (very sensitive or resistant) have been omitted from the plot to improve legibility. Inclusion of these values does not alter the pattern illustrated.

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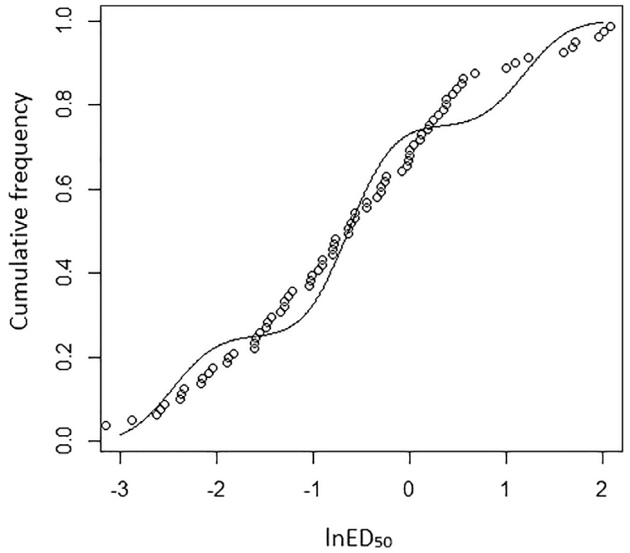

Figure 4. Comparison between the observed cumulative distribution of the $lnED_{50}$ values of the progeny of the AF28 × Spartan 1 cross (data points) and the expected distribution under the hypothesis that the difference between the parental isolates is due to two loci (continuous line). The sensitivity range and number of ascospores in the expected distribution are the same as in the observed data. A few of the most extreme values (very sensitive or resistant) have been omitted from the plot to improve legibility. Inclusion of these values does not alter the pattern illustrated.

Kolmogorov–Smirnov test $P = 0.005$). The two-gene (Fig. 4) and three-gene scenario (Fig. S2) were not excluded ($P = 0.3$; $P = 0.17$, respectively). However, seven progeny (9%) were more resistant than the resistant parent, Spartan 1, but 30 (37%) were more sensitive than the sensitive parent, AF28 (Fig. 2). This asymmetry is unlikely to have arisen by chance ($\chi_1^2 = 14.3$, $P < 0.001$), which excludes the no-epistasis hypothesis (H3).

4 DISCUSSION

The candidate resistant isolates in this study came from commercial orchards where a loss of scab control had been observed. The present result points to a very clear separation between the $lnED_{50}$ values of the baseline isolates and the candidate resistant isolates, with the latter generally having greater values. Although other factors may have contributed to the loss of scab control in these orchards, the presence of isolates with such high ED_{50} values is certain to have contributed. These results are in line with the trend of reduced sensitivity to myclobutanil observed in other countries.[2-7]

Tebuconazole was intrinsically somewhat more active than myclobutanil in inhibiting *V. inaequalis* colony expansion because the ED_{50} in the baseline population for myclobutanil was greater than for tebuconazole. There are discrepancies between the baseline values obtained in this study and those from previous studies. For myclobutanil, these values are greater in the current study than those reported previously in the UK with the single-spore *in vitro* assessment, with the CI for the current study 1.2–-4.5 mg L^{-1}, compared to 0.24 mg L^{-1}.[1] For tebuconazole, a study in Germany[26] found a baseline mean ED_{50} for tebuconazole (1.22 mg L^{-1}) higher than the one reported in the current study (95% CI 0.34–1.1 mg L^{-1}; $P = 0.05$).

Some of this variation could be due to chance variation between samples either spatially or with time. However, the current study differed slightly from both the previous studies, in distinct ways. Kunz *et al.*[26] used *in vivo* methods which often result in greater ED_{50} values than *in vitro* methods. In addition, the *in vivo* method they used involved the use of spore suspensions from lesions found on leaves, and posterior evaluation of spore differentiation in lesions from leaves inoculated with these spore suspensions; this means that the lesions examined may have contained a range of genotypes, resulting in the higher average ED_{50} values.

Xu *et al.*[1] worked with the diameters of single spore colonies, as in the present study. However, they and many other *in vitro* studies[6,23,27,28] used growth of the colony relative to the control (RG), whereas we used the actual mean growth at each concentration of chemical as response. We consider this preferable because dividing the growth by that of the control both introduces the error associated with the control into every measurement and loses a degree of freedom in the error estimate. This difference in approach would affect the residual error in the ED_{50} estimates but not the mean.

The distribution of baseline sensitivity in both myclobutanil and tebuconazole fitted a lognormal distribution well. Other authors found similar distributions of sensitivity to myclobutanil in baseline populations.[1,24] Some studies used one dose rather than a range in the sensitivity tests.[22,28,29] Using a single discriminatory dose is helpful where the sensitivity distribution is clearly bimodal, because the classification of an isolate is unchanged as long as the cut-off is kept between the two modes. However, in other types of distribution, the cut-off point can greatly alter how many isolates are reported as resistant. Although a single cut-off may increase the sample size in a given study, use of explicit estimates of ED_{50}, even with a wide CI, will improve comparability between studies.

The present results imply that tebuconazole would not be a good long-term replacement for myclobutanil, as their $lnED_{50}$ values correlate very closely; this is more evident in the baseline isolates. The observed cross-resistance may be due to similarities in the genetic control of the resistance to the two chemicals. Other studies also found greater cross-resistance to DMI fungicides in baseline rather than in exposed populations of *V. inaequalis* and other pathogens.[7,30]

It could assist the management of resistance to understand the genetic control behind it in a particular pathogen. The frequency distribution of the resistance to tebuconazole in the progeny of the cross AF28 × Spartan 1 shows that the difference in sensitivity in the parents is unlikely to be due to a single gene. Furthermore, the frequency distribution of sensitivity in the progeny was asymmetrical and transgressive segregation was observed, with progeny isolates significantly more resistant than the resistant parent, and more commonly, significantly more sensitive than the sensitive parent. This asymmetry is inconsistent with purely additive effects of the alleles at the loci involved and suggests the existence of epistatic effects; because *V. inaequalis* is a haploid organism, any role for dominance is only relevant if a locus is duplicated. In additive scenarios, progeny with $lnED_{50}$ values beyond those of the parents also would appear, but would be symmetrical, with similar proportions on both sides (Supplementary Information).

More work would be needed to shed light on what genes are involved in the control of resistance. In the cross in this study, both parents had their *CYP51A1* gene sequenced and no mutations could be found within the gene,[31] although the upstream region could not be included,[31] so *CYP51A1* could still be involved through an overexpression mechanism.

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The present findings regarding tebuconazole could help explain why the resistance to DMI fungicides has seemed to evolve more slowly than the resistance to other fungicide groups associated with single-gene resistance.[2,4,32] This could be due to more changes being necessary in order to get high levels of resistance.[33] In this type of multilocus evolution, all intermediate steps need to be advantageous; thus, if at one point a step involves a fitness penalty, a delay in natural selection occurs as a compensatory mutation is needed before the next change can take place. All of this does not rule out the existence of nonpolygenic forms of DMI resistance in *V. inaequalis* as other chemical families, such as the strobilurins, have demonstrated that both types of resistance can be found in the same pathogen.[34] However, when the two pathways exist in the same pathogen, the single-gene option is likely to emerge more commonly for two reasons: first, it may involve less danger of incurring a fitness penalty because only one change is necessary to confer resistance; second, the selection pressure per locus is greater. In general, this may be why there are more cases of single-gene resistance in plant pathogens.[35] However, it also is true that resistance controlled by a single locus is easy both to detect and to study.

5 CONCLUSIONS

The findings of this study mean that, in the long term, other chemicals would have to be found or developed to be used in place of myclobutanil where resistance is a problem. In the short term, tebuconazole may be used if recommended doses are still effective on a myclobutanil-resistant apple scab population.

The distribution of $lnED_{50}$ values of the progeny of the resistant x sensitive isolate cross gives some hints about the genetic architecture controlling sensitivity. Further analysis and examination of other crosses, in combination with sequence or marker data, could elucidate variation in sensitivity to tebuconazole and identify relevant genes. This may enable the design of disease management programs to take into account the development of fungicide resistance, prolonging the lifetime of fungicides.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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