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Effect of azole fungicide mixtures, alternations and dose on azole sensitivity in the wheat pathogen *Zymoseptoria tritici*

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Abstract

The evolution of fungicide resistance in the cereal pathogen *Zymoseptoria tritici*, is a serious threat to the sustainability and profitability of wheat production in Europe. Application of azole fungicides has been shown to affect fitness of *Z. tritici* variants differentially, so it has been hypothesised that combinations of azoles could slow the evolution of resistance. This work was initiated to assess the effects of dose, mixtures and alternations of two azoles on selection for isolates with reduced sensitivity and on disease control. Naturally infected field trials were carried out at six sites across Ireland and the sensitivity of *Z. tritici* isolates monitored pre- and post-treatment. The azoles epoxiconazole and metconazole were applied as solo products, in alternation with each other and as a pre-formulated mixture. Full and half label doses were tested. The two azoles were partially cross-resistant, with a common azole resistance principal component accounting for 75% of the variation between isolates. Selection for isolates with reduced azole sensitivity was correlated with disease control. Decreased doses were related to decreases in sensitivity but the effect was barely significant (P = 0.1) and control was reduced. Single applications of an active ingredient (a.i.) caused smaller decreases in sensitivity than double applications. Shifts in sensitivity to the a.i. applied to a plot were greater than to the a.i. not applied, and the decrease in sensitivity was greater to the a.i. applied at the second timing. These results confirm the need to mix a.i.s with different modes of action.

Introduction

Septoria tritici blotch (STB) caused by the ascomycete pathogen *Zymoseptoria tritici* Quaedvl. & Crous, (synonym: *Mycosphaerella graminicola*) is the main disease of winter wheat grown in Europe (Cools & Fraaije, 2013). If left unchecked it has the potential to reduce yields of susceptible cultivars by anything up to 50% (Burke & Dunne, 2008).

Cultural control practices, e.g. late sowing, can be used to reduce the damage caused by STB (Shaw & Royle, 1993), but can limit yields themselves (Green & Ivins, 1985). Whilst host resistance is available it often imposes yield penalties and therefore commercially available wheat cultivars are likely be high yielding but only moderately resistant to diseases such as STB (Brown, 2002). Control of STB is therefore currently largely reliant on the timely application of fungicides. Unfortunately the development and widespread occurrence of resistance to the quinone outside inhibitor (QoI) and methyl benzimidazole carbamate (MBC) classes of fungicides in European *Z. tritici* populations has reduced the number of effective groups of fungicides available for STB control (Fraaije *et al*., 2005). Those now available include the multisite inhibitors such as chlorothalonil and folpet which act as protectant only and the succinate dehydrogenase inhibitors (SDHIs) and sterol 14α-demethylation inhibitors (DMIs) which provide protectant and eradicant activity. The development of insensitivity in *Z. tritici* to these remaining chemistries poses a threat to the future control of STB in Europe. Whilst the multisite inhibitors are at a low risk of resistance, the risk to DMIs has been classified as medium (Brent & Hollomon, 2007) and the risk of resistance to the SDHIs in *Z. tritici* is regarded as high (Fraaije *et al*., 2012). Therefore, the development of anti-resistance strategies to prolong the effective life of both these groups of fungicides is much needed.

The DMI fungicides have been widely used in cereal production since the 1980s (Hollomon *et al*., 2002). The azoles, largely represented by the triazoles but also including the triazolinthione derivative prothioconazole and the imidazole prochloraz, are the main chemical group within the DMI class. Since the development of resistance to the QoIs, the azoles have been the backbone of STB control in winter wheat (Fraaije *et al*., 2007). For more than a decade, a progressive reduction in sensitivity to azoles has been observed in European *Z. tritici* populations (Stammler & Semar, 2011). This reduction in sensitivity has been attributed to a number of different mechanisms, including amino acid alterations in the

target site (14α-demethylase or *CYP51*), overexpression of the target site, and, perhaps, increased efflux of the fungicides (Cools & Fraaije, 2013). Since the early 1990s, alterations in the *CYP51* gene have been identified, many of which had only slight effects on sensitivity to the majority of azoles (Cools & Fraaije, 2013). However, these early alterations may have, over the past 10-15 years, facilitated the emergence of alterations which affect the binding of specific azoles, leading to a reduction in sensitivity (Mullins *et al*., 2011). Many of these changes can alter the sensitivity to specific azoles differently, as highlighted by Fraaije *et al*. (2007). For example, the now common I381V mutation is strongly selected by both tebuconazole and metconazole but the same mutation is selected against by the imidazole, prochloraz. The V136A mutation, however, makes *Z. tritici* more sensitive to tebuconazole but less sensitive to prochloraz (Fraaije *et al*., 2007). Since 2008, isolates of *Z. tritici* with reduced sensitivity to epoxiconzole and prothioconazole have become common in Ireland. While these variants have predominantly had the *CYP51* alterations V136A and S524T (Stammler & Semar, 2011), and even though the S524T mutation has the effect of reducing sensitivity to many azoles (Cools *et al*., 2011) sensitivity to metconazole and tebuconazole has been maintained (O'Sullivan & Kildea, 2010). This apparent lack of cross-resistance suggests that using multiple azoles in combination, either as mixtures or sequentially, may provide a means of decreasing selection for isolates with reduced sensitivity while maintaining disease control (Cools & Fraaije, 2013).

Evolution of resistance can be divided into three phases (Van den Bosch *et al*., 2011). In the emergence phase, resistance arises due to mutation and/or invasion. The selection phase occurs when the resistant isolates increase as a proportion of the whole population due to the application of fungicide. Finally, in the adjustment phase, resistance is so common that agronomic practices need to be adjusted to deal with it (Van den Bosch *et al*., 2011).

Management of resistance during each phase may differ, but this paper focusses on the selection phase.

Combinations of fungicides intended to slow down the selection of resistance usually involve fungicides with different modes of action. However, due to the commercial preference for fungicide products with activity against multiple fungal targets, combining azoles has become increasingly common in fungicide programmes on winter wheat. Unfortunately, not much is known about how such combinations alter the evolution of *Z. tritici* sensitivity. Most of the few sources of empirical data available for azole mixtures measured only STB control (Kendall & Hollomon, 1994, Kendall et al., 1996, Du Rieu et al., 1994), rather than the impact on *Z. tritici* sensitivity. A single report included azole mixtures (imidazole and triazole fungicides) in the context of resistance management (Fraaije *et al*., 2011). It suggested that using mixtures of azoles which differentially select specific mutations can lead to a decrease in the frequency of isolates with reduced sensitivity, but it depends on the components of the mixture. Similarly, there is very little empirical information available on how alternations of azoles affect selection for isolates with reduced azole sensitivity. Hobbelen *et al*. (2013) reviewed models which study the effects of mixtures and alternations as anti-resistance strategies and found that most were designed to study combinations of low- and high-risk fungicides. None of these models discussed in depth the mixing or alternation of fungicides which target the same site.

In addition to the above recommendations, the reduction of fungicide dose has been suggested as an anti-resistance strategy (van den Bosch et al., 2014), particularly in the selection phase. However, where isolates with reduced sensitivity are present in a large proportion in the population, reducing the recommended dose per application is likely to lead to a reduction in disease control, potentially making such a strategy impractical (Hobbelen *et al*., 2011a).

The aim of the work reported here was to test the following hypotheses. Firstly, that combinations of azoles, either in mixtures or alternated at different application timings, will slow the rate at which reduced sensitivity to either active ingredient is selected in field populations of *Z. tritici*. Secondly, lower than recommended doses at each application will decrease selection for isolates with reduced sensitivity. To test these hypotheses, field trials using commercially available products in high disease pressure environments were combined with sensitivity testing of *Z. tritici* isolates sampled pre- and post-fungicide application.

Materials and methods

Trial design and fungicide application

Field trials were conducted during 2010-11 and 2011-12 at six locations in wheat-growing areas of Ireland (Table 1). All trials were laid out as complete randomised block designs with four replicate blocks, each containing 10 fungicide treatments and an untreated control. Plots were 2.5m × 10m with a 30-40cm path between plots. *Zymoseptoria tritici* was allowed to develop naturally in each trial. Experimental treatments consisted of two foliar fungicide applications (referred to as T1 at GS 32-37 and T2 at GS 39-53 depending on site (Zadoks *et al*., 1974)) of the azoles epoxiconazole (Opus®, BASF) and metconazole (Caramba®, BASF) as solo products, in alternation with one another at the different timings or as a mixture of both (Gleam®, BASF), and all of the above at full and half the recommended dose (see Table 2 for further details). Both fungicides were widely used in Ireland. All fungicides were applied in 200L/ha water using a knapsack sprayer with compressed air.

Disease and yield assessments

Disease was assessed at GS 69-73 on the flag leaf of ten main tillers chosen at random, at approximately equal distances apart in each plot. The flag leaf was assessed because it has

most influence on yield. The percentage leaf area with STB was visually estimated. Plots were harvested each year using a specially adapted combine harvester. The grain from each plot was weighed and the moisture content determined in a representative sample from each plot. Yields were then calculated as t/ha at 15% moisture.

Sampling *Zymoseptoria tritici*

To determine the distribution of fungicide sensitivity in the *Z. tritici* population prior to spraying, each site was sampled. In 2011 approximately 100 diseased leaves and in 2012 approximately 50 diseased leaves were collected from each of the trial sites, sampled uniformly from across the whole site. At the second sampling time (six weeks post T2 fungicide application), approximately 40 diseased flag leaves were collected, without regard to disease severity, at roughly equal distances apart within each plot. The flag leaf was chosen because it represents the close to final reproducing population on the crop. At the Stamullen and Knockbeg sites in 2011, disease levels were too low six weeks after T2 so sampling was conducted eight weeks after the T2 fungicide application. At these two sites, disease levels were still low after eight weeks and diseased leaves were actively sought. The diseased leaves from each plot were air dried for five days at room temperature and then stored at -20°C awaiting pathogen isolation.

Isolating *Zymoseptoria tritici*

Isolations were carried out according to Kildea (2009). Briefly, diseased leaves (cut to fit four in a 10cm petri dish) were washed in running tap water for two hours before being surface sterilised (immersed in 70% ethanol for 20 seconds, 10% sodium hypochlorite for 2 minutes and triple rinsed with sterile distilled water). The leaves were subsequently dried using tissue paper and placed, exposed pycnidia facing upwards, on water agar, then incubated in the dark at 18°C for 24-48 hours to promote sporulation. Following incubation, a single cirrus from each leaf was picked using a fine sterile needle and streaked onto potato glucose agar (PGA)

(Sigma-Aldrich, St. Louis, MO, USA) amended with 50mg/l chloramphenicol and 50mg/l streptomycin. Petri dishes were sealed and incubated in the dark at 18°C for 4-6 days. Isolates were sub-cultured onto antibiotic amended PGA (as above), sealed and incubated at 18°C for a further three days. Pure cultures were scraped from the plates and individually stored in 30% glycerol at -80°C until further use.

In-vitro sensitivity testing

The sensitivity of all isolates to epoxiconazole and metconazole was determined using a microtitre plate assay as described by Kildea (2009). Briefly, technical grade epoxiconazole and metconazole (purchased from Sigma-Aldrich Co.) were dissolved in 100% methanol and added to Potato Dextrose Broth (PDB) (Sigma-Aldrich Co.) to give final test concentrations of 30, 10, 3.3, 1.1, 0.37, 0.123, 0.04, and 0 mg/l of which 150µl was added to wells of flat bottomed sterile 96-well microtitre plates (Sarstedt AG & Co., Germany). Inoculum of each isolate was produced by spotting 30µl of the stock solutions stored at -80°C on PGA and incubated for three days at 18°C. Test suspensions were made in PDB and adjusted to a final concentration of $1x10^5$ spores/ml, 50µl of which was added to the wells of the microplates containing the different fungicide concentrations. Each plate consisted of a negative control (PDB only), a positive control (isolate 4465, of Irish origin and kindly supplied by BASF) and 10 experimental isolates. In some exceptional cases, isolate 4465 did not produce sufficient spores to allow for the inclusion of a positive control in all test plates. Two replicate plates were tested at the same time, sealed with parafilm, stored in sealable bags to reduce condensation and incubated in the dark at 18°C for 7 days. Due to the large number of isolates in the whole experiment, isolates from the same plot, field replicate, site or treatment were not necessarily tested on the same date. Fungal growth was assessed as a measure of light absorbance at 405nm using Synergy-HT plate reader and Gen5™ microplate software (BioTek Instruments, Inc., USA).

Statistical analysis

The fungicide dose reducing growth in the microplate wells by 50% (EC_{50}) was determined by fitting a logistic curve to percentage inhibition data generated from the optical density measurements for each isolate using XLfit (IDBS Inc., UK). Where a plate had a reference isolate, EC_{50} values from that plate were adjusted for differences in the reference isolate between plates according to Mavroeidi and Shaw (2005). The subsequent analysis was weighted to allow for the increased variance of observations from plates where the EC_{50} of the reference isolate could not be measured. Observations from plates with a successful reference isolate measurement were given a weight of 1 and a value of 1-(variance within the standards/variance in isolates from plates with standards) given otherwise. All statistical analyses were carried out in GenStat 14th Edition (VSN International Ltd. United Kingdom). Differences between plate replicates were analysed using ANOVA. As the numbers of isolates with successfully measured EC_{50} values varied between plots, the data were not balanced. Treatment differences were therefore analysed and means constructed using Restricted Maximum Likelihood (REML). Data from the early sampling time (Pre-T) were analysed using REML, whilst data from the later sampling time were analysed using REML with contrasts (Crawley, 2005), using the FCONTRASTS procedure. Contrasts were constructed to specifically test the hypotheses in the model (Table 3), with 4 additional contrasts included in the analysis but not shown because of non-significant results. Each contrast - a weighted comparison between a set of means, with weights adding to zero - represents a single degree of freedom in the data. Contrasts were constructed to be as independent as possible from the other contrasts; in fully balanced data they would be completely independent. This means that the significance tests for each hypothesis examined were independently valid. In the model, treatment (11 levels) was considered a fixed effect, whilst site (six levels) and replicate (four levels) and site.treatment were considered random

effects. Contrasts were estimated separately for sensitivity to epoxiconazole and metconazole. Principal Components Analysis (PCA) was used to determine the common effects of using epoxiconazole and metconazole on overall sensitivity, and to look at how selection by epoxiconazole and metconazole affected specific resistance to each active ingredient (a.i.). Sensitivity data were subjected to PCA based on sums of squares and products. Principal component scores, PC1 and PC2, were analysed using REML with contrasts.

Disease severity data were square root (sqrt) transformed and differences between treatments were analysed using ANOVA with a factorial plus control procedure. Disease severity data were correlated with the sensitivity data using general linear regression including differences in sensitivity between sites as a factor. Differences in yield were analysed using ANOVA with a factorial plus control procedure and the relationship between yield and disease control was estimated using general linear regression including site differences, but assuming a common slope.

Results

The sensitivity of 3707 single pycnidial *Z. tritici* isolates were determined. Due to contamination or no growth of some isolates on some plates, 3703 were tested for sensitivity to epoxiconazole, and 3683 isolates were tested for sensitivity to metconazole (Table 3). Sensitivity data were not determined for the half-dose alternation treatments in 2011-12. There was no statistical difference (P = 0.9) between replicate plate measurements of each isolate and therefore mean EC_{50} values for each isolate were used in the subsequent analysis.

Variability before fungicide applications

Isolates from the population prior to fungicide applications (Pre-T) ranged in sensitivity to epoxiconazole from a $\log_{10}EC_{50}$ (mg l^{-1}) of -2.38 to 0.51 (a ratio of 776), and to metconazole from a $\log_{10}EC_{50}$ (mg l^{-1}) of -2.38 to 1.35 (a ratio of 5370) (Fig. 1). At this sampling time

sensitivity to epoxiconazole was similar at all sites (Fig. 1a, $P = 0.15$), but sensitivity to metconazole differed between sites (Fig 1b, $P < 0.001$).

Main contrasts

Main effects rather than specific differences between the effects of particular treatment patterns at individual sites and years of observation would be relevant to the choice of overall resistance management strategy, so main effect contrasts are reported, using site and site interactions as random factors in the mixed effect REML model. *Zymoseptoria tritici* isolates sampled from treated plots were less sensitive to both epoxiconazole and metconazole than isolates from the untreated plots ($P < 0.001$ and $P < 0.001$ respectively, Table 4, contrasts 1a and 1b) with large reductions in sensitivity at some sites, for example at Duleek, Julienstown and Killeagh, there was a two to four-fold decrease in sensitivity to epoxiconazole and at Stamullen, a 44-fold decrease was observed (Table 3a). All treatments containing epoxiconazole selected more for reduced sensitivity to epoxiconazole than those treatments with none ($P < 0.001$, Table 4, contrast 2a). The same was seen for sensitivity to metconazole, where all treatments containing metconazole selected more than treatments without metconazole ($P = 0.002$, Table 4, contrast 2b). There was no significant difference between the effect of the mixture and the solo epoxiconazole on sensitivity to epoxiconazole ($P = 0.3$, Table 4, contrast 3a) or between the effect of the mixture and the solo metconazole on sensitivity to metconazole ($P = 0.42$, Table 4, contrast 3b). *Zymoseptoria tritici* isolates from treatments which received two applications of epoxiconazole were less sensitive than those that received only one, although the difference was not quite significant ($P = 0.09$, Table 4, contrast 4a). Treatments which applied metconazole twice selected significantly more for sensitivity to metconazole ($P = 0.03$, Table 4, contrast 4b) than the treatments which applied metconazole only once. The order in which the a.i. was applied in the alternation had no effect on sensitivity to epoxiconazole ($P = 0.1$, Table 4, contrast 5a) or sensitivity to

metconazole (P = 0.9, Table 4, contrast 5b). Even though full doses tended to cause a slight increase in selection for isolates with reduced sensitivity to both fungicides (ns, P = 0.12, Table 3) averaged over all treatments the difference between half doses and full doses was not significant for sensitivity to either epoxiconazole or metconazole (P = 0.2 and P = 0.2 respectively, Table 4, contrasts 6a and 6b). Interactions between dose and contrasts 2-5 were tested but all were non-significant (data not shown).

Principal components analysis

The first principal component (PC1: a measure of common sensitivity to both epoxiconazole and metconazole) accounted for 75% of the total variation amongst the isolates (Fig. 2). The loadings for each variable were almost equal, meaning sensitivity to both epoxiconazole and metconazole made an almost equal contribution to the variation between isolates. PC1 differed significantly between the untreated and treated plots (P < 0.001, Table 5, contrast 1a) and between the solo products and the mixture (P = 0.002, Table 5, contrast 3a). All other contrasts, including the interactions between dose and contrasts 2-5, were non-significant. The second principal component (PC2: a measure of the distinction between sensitivity to epoxiconazole and metconazole) accounted for the remaining 25% of total variation (Fig. 2). PC2 differed between the solo a.i.s (P < 0.001, Table 5, contrast 2b). Also, the order of a.i.s in the alternation treatments affected selection on PC2 (P = 0.05, Table 5, contrast 5b) but this effect differed between doses (P = 0.01, data not shown). All other contrasts were non-significant.

Disease severity and its relationship with selection

Untreated control plots had the most disease at all sites (P < 0.001); with an average of 12% (3.46 sqrt %) disease severity on the flag leaf at GS 69-73 (Fig. 3). Significant differences in disease severity in the untreated plots were observed between sites (P < 0.001); Julienstown had the most disease in untreated plots, with 25% (4.964 sqrt %) of the flag leaf infested with

STB, and Stamullen had the least, with 0.3% (0.510 sqrt %). Significant differences in disease severity in the treated plots were observed between sites ($P < 0.001$); Stamullen had the least disease after treatment, with 0.05% (0.22 sqrt %) and Julienstown and Killeagh had the most, both with 3.5% (1.87 sqrt %) disease on the flag leaf. The full dose treatments controlled STB better than their half dose counterparts ($P = 0.015$). Disease control differed between treatments ($P < 0.001$); with the mixture providing significantly better disease control (0.78% disease severity (0.88 sqrt %)) than any of the other treatments (average 2.17% (1.47 sqrt %) disease severity). There was an inverse relationship between disease severity and sensitivity of isolates to epoxiconazole and metconazole (Fig. 4a, $R^2 = 0.48$, $P < 0.001$ and Fig. 4b, $R^2 = 0.60$, $P < 0.001$ respectively; common slope but intercepts differing between sites).

Yield

Untreated control plots yielded significantly less than treated plots ($P < 0.001$, Table 6). Yield improvements after fungicide application varied between sites ($P < 0.001$, Table 6). Oak Park, Duleek and Knockbeg each had an improvement of 2 t/ha after fungicide treatments whereas Stamullen had the lowest with an improvement of only 0.1 t/ha, consistent with the low untreated disease severity. Averaged over all treatments, full doses provided significantly higher yield than the half doses, and the half doses were significantly better than no fungicide ($P = 0.001$, Table 6). No differences in yield were seen between the two solo a.i.s, the two alternations or the mixture ($P = 0.17$, Table 6). There was a significant inverse relationship between disease and yield; but both the slope and intercept of this varied between sites (Fig. 5, $R^2 = 0.98$, $P = 0.014$).

Discussion

To prolong the life of fungicides, strategies which delay both emergence and selection of resistant strains, without compromising yield, are needed. In these experiments, where STB was the dominant disease, yields achieved were directly related to the control of disease and associated with greater selection for isolates with reduced sensitivity. This confirms the findings of Mavroeidi and Shaw (2006) who demonstrated that when the azole fluquinconazole was applied as a solo product, selection was positively correlated with control. In the current study, the increase in isolates with reduced sensitivity was proportional to the reduction in disease severity at each site, irrespective of the initial sensitivity of the population. Whilst the use of six sites with varying sensitivity to both epoxiconazole and metconazole presents difficulties in determining the effects of individual treatments, the results are a realistic representation of the response of the Irish *Z. tritici* population, which varies in sensitivity to azoles.

With high levels of phenotypic variation between isolates at each site early in the season, a wide base from which selection could occur was present. Irrespective of application pattern (solo, mixture or alternation) or dose, all fungicide treatments significantly decreased the sensitivity of *Z. tritici* to both epoxiconazole and metconazole. The presence of cross-resistance between both azoles tested, as identified in the PCA, explains this common effect of fungicide treatment on sensitivity. Conversely, the PCA did highlight that this cross-resistance was not complete, with 25% of the variation amongst the isolate collection resulting from differences between the azoles. This may have contributed to the results from the REML and PCA which showed that each fungicide differentially selected. Even though epoxiconazole and metconazole target the same protein, earlier evidence showed that different azoles select for different *CYP51* genotypes (Fraaije et al., 2007, O'Sullivan & Kildea, 2010, Stammler & Semar, 2011). There is evidence of considerable evolution in the

CYP51 gene (Cools & Fraaije, 2013) and recent work has identified *CYP51* alterations which can reduce sensitivity to the majority of azoles, in particular the S524T mutation (Cools *et al*., 2011) and the V136A + I381V combination (Stammler *et al*., 2008), as well as strains which overexpress the target gene (Cools *et al*., 2012).

Recent theoretical modelling of the potential emergence and subsequent selection of resistant or partially resistant strains (Hobbelen *et al*., 2013, Hobbelen *et al*., 2014, Mikaberidze *et al*., 2014, Van den Bosch *et al*., 2011) predicts that mixtures of fungicides, whether high-risk:high-risk or high-risk:low-risk combinations, will prolong the effective life of the most at-risk partner. Unlike these models, our experiments used a mixture of fungicides with medium-resistance-risk and belonging to the same chemical class. Mixtures expose a pathogen population to different modes of action, albeit simultaneously rather than sequentially as with alternations. Each component of a mixture should control a proportion of the isolates selected by the other component, thereby reducing the overall selection compared to using a single fungicide. When the effects of treatments on sensitivity were studied for each a.i. separately, the expected positive effect of mixing two components was not seen. Further, when the effects common to both epoxiconazole and metconazole sensitivity were analysed using PCA, the mixtures were seen to select significantly more than the solo treatments. This increase in selection by the mixture, which contained 90% of the solo epoxiconazole dose and 92% of the solo metconazole dose, could simply be due to a further dose effect (Fig. 6). Alternatively, interactions between the fungicides in the mixture are likely to have some effect on both disease control and selection. Synergism between the fungicides could explain the improvement in disease control (Kendall & Hollomon, 1994) and the absence of a reduction in selection (Shaw, 1993). Shaw (1993) suggested that such synergism could be used to reduce selection by using the minimum fungicide dose needed for adequate control, however, in these results the half-dose of the mixture gave almost as much

control as the full dose, so is not the minimal effective dose . Like the alternation treatments, this mixture does not conform to those usually prescribed for anti-resistance purposes, as reviewed by van den Bosch *et al*. (2014) and van den Bosch *et al*. (2014b); it is a mixture of two azoles which display a moderate to high level of cross-resistance.

Limiting the number of applications of an a.i. decreased the selection of isolates which were less sensitive to that a.i. In the treatments where only one application of metconazole was made, the population was significantly more sensitive than the treatments where two applications were made. Even though this was just non- significant at the 5% level for sensitivity to epoxiconazole (P = 0.09), the same pattern was seen. This supports the fungicide resistance model by Hobbelen *et al*. (2011b) in which a significant increase in the selection ratio with an increase in spray numbers was predicted. Increasing the time span whereby a fungicide is in contact with the pathogen increases the fitness advantage of those strains able to survive in its presence, resulting in resistance build up (van den Bosch *et al*., 2014). Applying the same a.i. at each treatment time i.e. solo treatments and mixtures, increases that time span. Alternations on the other hand allow time between applications of the same fungicide for back-selection of susceptible strains. It could be argued that the alternation treatments in this study do not reduce this time span as strong cross-resistance was evident between the fungicides, however, PC2 of the PCA demonstrated the benefit of the alternation treatments. Isolates from plots treated with metconazole first and epoxiconazole second were less sensitive to epoxiconazole, and those from plots treated with epoxiconazole first and metconazole second were less sensitive to metconazole i.e. the most recently applied fungicide had the greatest effect on selection. No comparable findings in an agricultural setting are available. The model of Hobbelen *et al*. (2013) included alternations of fungicides with different modes of action and predicted it would delay the selection of strains with reduced sensitivity. While different sequences of fungicides were included in the model, this

effect of the order of fungicide was not predicted. The model was based on selection within the entire upper canopy and not just a single leaf layer subjected to only the most recent fungicide, as described in this study. If looking at selection from season to season i.e. the inoculum left to infect the following crop, then using the whole canopy seems sensible and order of use probably would not have a significant effect. On the other hand, the current data indicated that selection within season and the inoculum most likely to contribute to epidemic progress i.e. that on the uppermost leaf which received the last treatment, may well be affected by order of use and may affect disease control..

Although there was a trend for the half doses to decrease selection, averaged over all application strategies this decrease was not significant. As expected, full doses provided significantly better disease control and, where there was high disease pressure, higher yields. While full doses of fungicide are designed to provide the best possible disease control and are recommended as an anti-resistance strategy by manufacturers, Van den Bosch *et al*. (2011) reviewed the available literature and concluded that all models and most experimental studies show that selection of strains with reduced sensitivity increases with dose. However, the same study highlighted that where insensitivity develops gradually, such as *Z. tritici* insensitivity to DMIs, there may be exceptions to this rule. They suggested that in this case it is possible that the dose response curves of the sensitive strains and less sensitive strains converge within the permitted dosage, reducing the fitness advantage of the less sensitive strains; in this case higher doses may actually reduce selection. It is possible that the response curves of the majority of isolates in this study converge at the maximum doses used, leading to a reduction of the overall fitness advantage of the insensitive strains and modest levels of selection. The plateauing of sensitivity following treatment with half and full rates of the mixture may be a further example of convergence. It is, however, probable that the population examined is in fact in the adjustment phase of resistance evolution, where the

minimum rates of azoles required for effective control are now larger than before. Inferior disease control and lower yields makes reducing rates of azole fungicides impractical when they are used alone, but in cases of diseases which are in the selection phase of resistance development, the minimum rates required for effective control may be lower, and using lower rates is a practical strategy for reducing selection. The contrast representing comparison of doses was made within a product type; it is possible that the mixture comparison was in the plateau of the dose response curve, reducing the size and significance of the overall contrast.

Where only combinations of azoles are used, it seems that limiting the number of applications of an individual a.i. is the most important strategy for managing azole sensitivity; having two azoles which select differentially, and using each sequentially rather than simultaneously, will slow down the selection of strains with reduced sensitivity to those azoles. Additionally, and essential for resistance management, while disease control achieved by the alternations was the same as that of solo products and control by both were poorer than the mixtures, the yields were not significantly different. This strengthens the case for choosing azole alternations over azole mixtures or solo azole a.i.s, and emphasises that aiming for perfect disease control may incur costs and increase selection without increasing output. But in the long term, azole combinations are probably unsustainable. The cross-resistance observed makes long term benefits from using combinations of azoles unlikely, and highlights the need for the inclusion of alternative chemistries in fungicide programs. However, the individual components of a mixture should be effective in their own right; otherwise they do not protect the other component. Our results demonstrate that anti-resistance recommendations for fungicides with distinct modes of action are not always effective when using combinations of azoles, and advice to combine azoles which select for different resistance alleles or loci is vulnerable to continuing genetic change in the pathogen.

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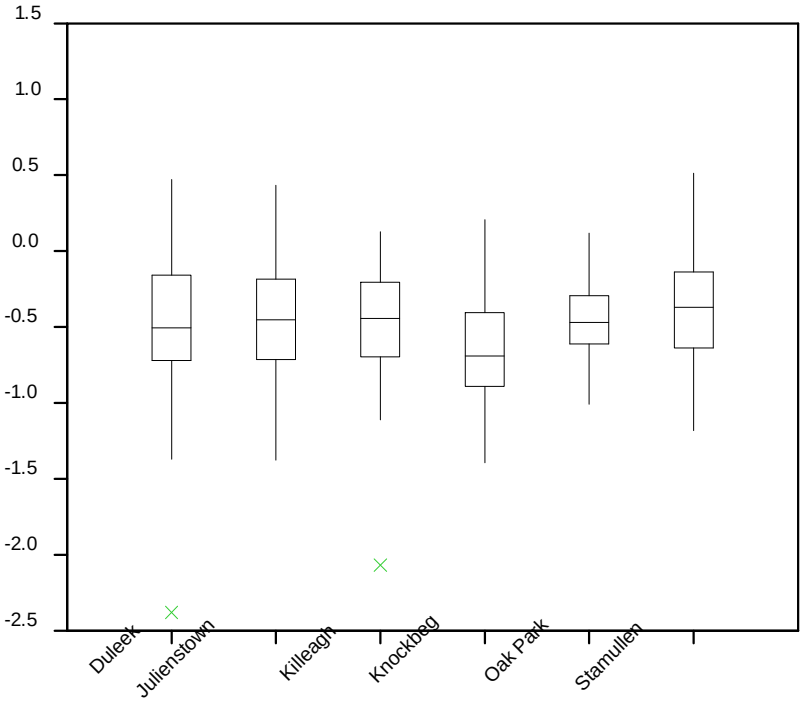
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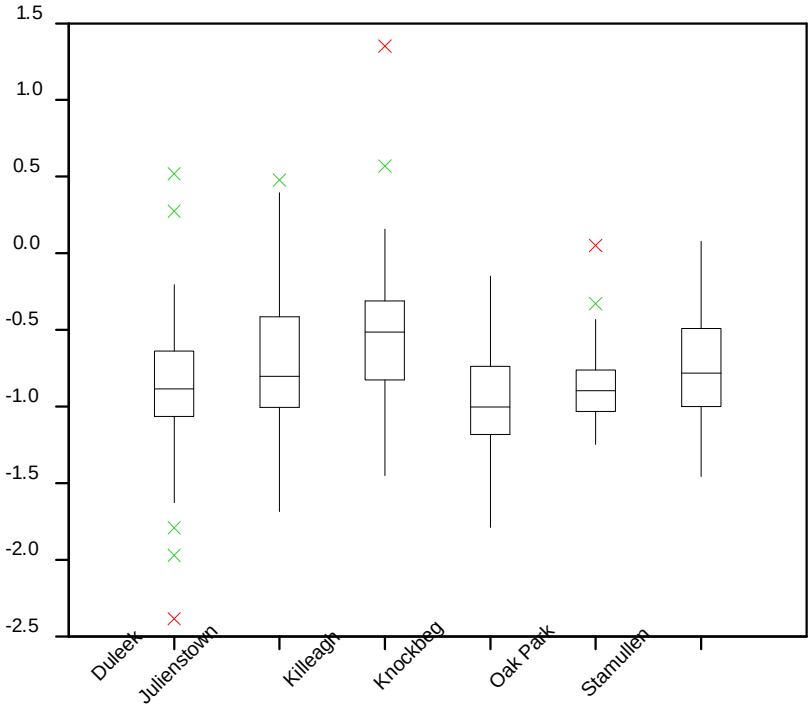
Oak Park
Stamullen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Knockbeg
Oak Park
Stamullen

Figure 1 Frequency distribution of log EC_{50} values for sensitivity to epoxiconazole (top) and sensitivity to metconazole (bottom) from Pre-T collections of *Zymoseptoria tritici* sampled from each of the six sites, illustrated with box and whisker plots. The line through the box represents the median. The crosses represent outliers. Number of Pre-T isolates tested from each site; Duleek n = 33; Julienstown n = 29; Killeagh n = 20; Knockbeg n = 25; Oak Park n = 21; Stamullen n = 48. Sensitivity to epoxiconazole did not differ between sites ($P = 0.15$) whereas sensitivity to metconazole did ($P < 0.001$).

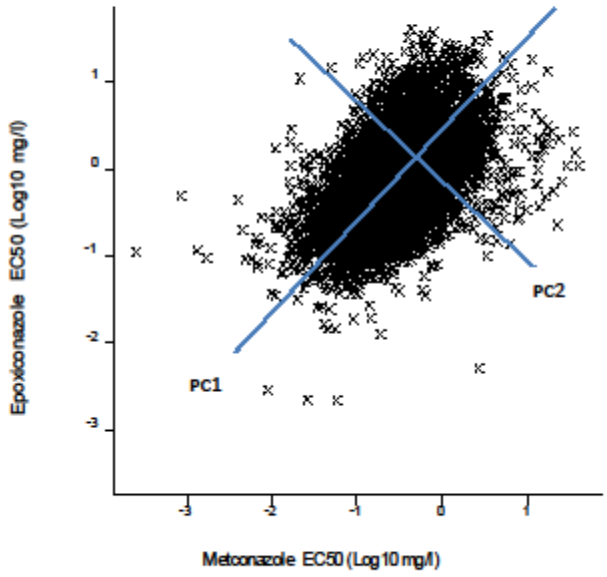

Figure 2 Correlation matrix with Principal Component axes superimposed. PC1 accounts for 75% variation, PC2 accounts for 25% variation.

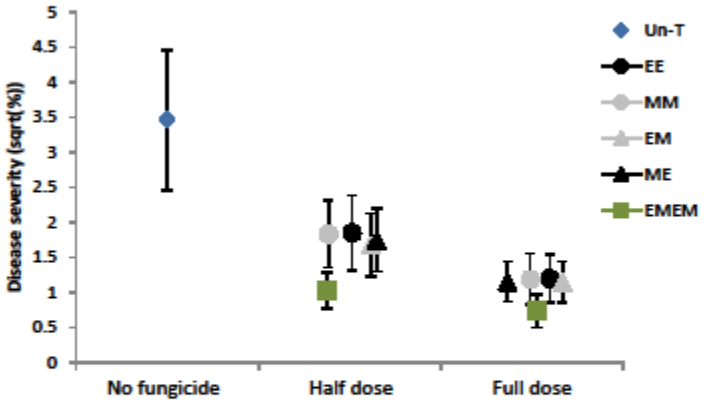

Figure 3 Effect of individual treatments on disease severity on the flag leaf at GS 69-73 averaged over all six sites. Disease severity refers to the proportion of the flag leaf covered in Septoria tritici blotch (square root transformed). Error bars are 1 SED. Treatment information: abbreviations denote the first and second sprays. E: epoxiconazole; M: metconazole; Un-T: untreated control.

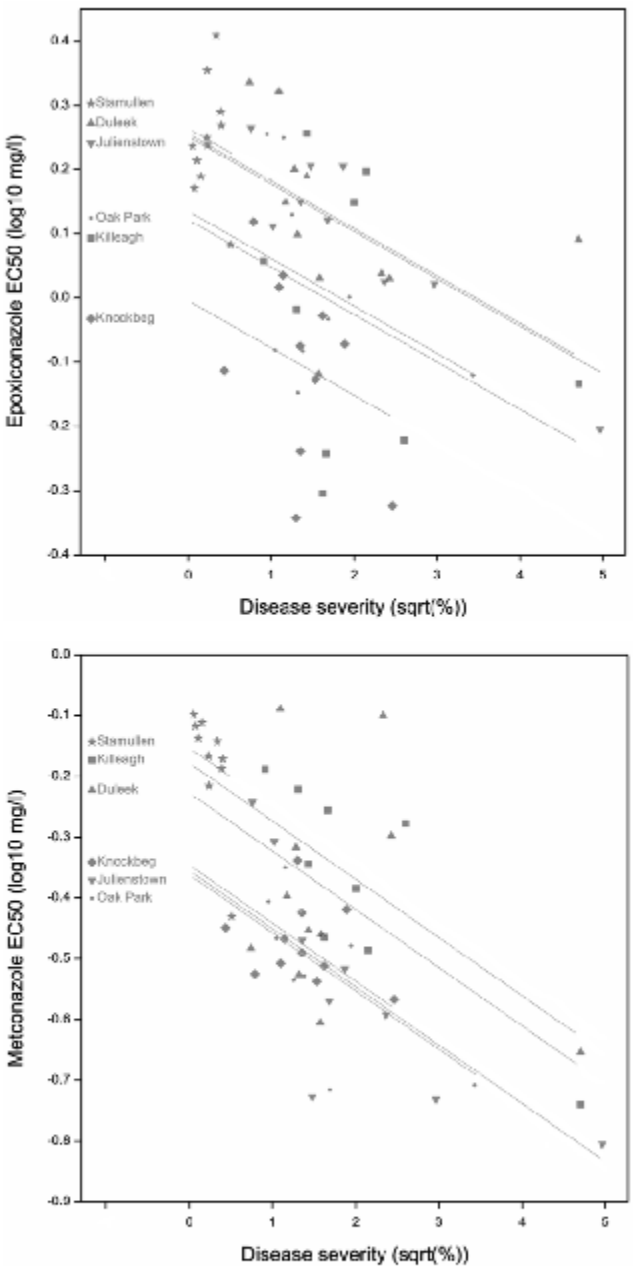

Figure 4 Fitted and observed relationship between (a) sensitivity to epoxiconazole and disease severity, $R^2 = 0.48$, $P < 0.001$; common slope = -0.074; intercept for Duleek = 0.256; Julienstown = 0.252; ; Killeagh = 0.122; Knockbeg = -0.004; Oak Park = 0.135; Stamullen = 0.264 and (b) sensitivity to metconazole and disease severity $R^2 = 0.60$, $P < 0.001$, common slope = -0.096; intercept for Duleek = -0.227; Julienstown = -0.355; Killeagh = -0.177; Knockbeg = -0.345; Oak Park = -0.361; Stamullen = -0.153. The topmost regression line corresponds to the topmost site, labelled on the left of the graph, and so on down.

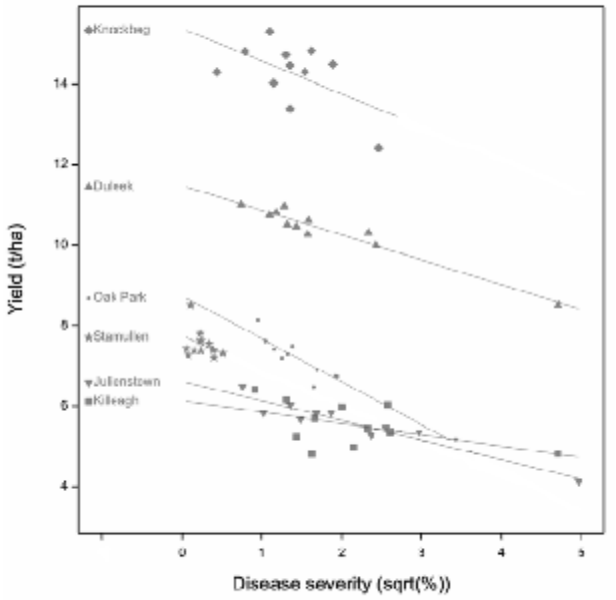

Figure 5 Fitted and observed relationship between yield and disease severity $R^2 = 0.98$, *P* = 0.014; Duleek: Y = 0.208 + -0.047X; Julienstown: Y = -0.102 + -0.308X; Killeagh: Y = -0.046 + 0.065X; Knockbeg: Y = -0.13 + 0.073X; Oak Park: Y = -0.094 + 0.167X; Stamullen: Y = 0.022 + 0.24X. The topmost regression line corresponds to the topmost site, labelled on the left of the graph, and so on down.

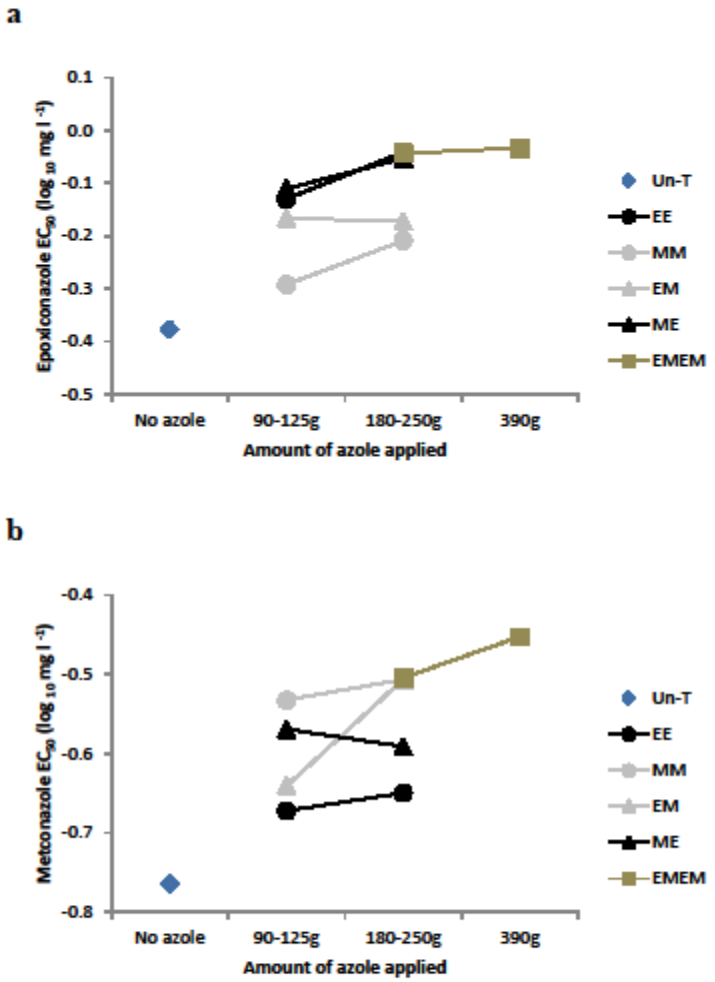

Figure 6 Effect of total azole dosage on the sensitivity of isolates to epoxiconazole (a) with an average SED of 0.069 and metconazole (b) with an average SED of 0.07. Treatment information: abbreviations denote the first and second sprays. E: epoxiconazole; M: metconazole; Un-T: untreated control.

Table 1 Site details, year each site was included, timing of fungicide applications and growth stage at which fungicides were applied (GS)

Site (Coordinates)	*Year*	*Cultivar*	*Septoria resistance rating* [a]	*Date of first application (T1)*	*GS* [b] *at T1*	*Date of second application (T2)*	*GS* [b] *at T2*	*Date of disease assessment*	*GS at disease assessment*
Duleek (53.673502, -6.374087)	2011	Cordiale	4	28th April	33	19th May	51	27th June	71
Julienstown (53.679806, -6.309156)	2012	Cordiale	4	3rd May	33	29th May	39	26th June	73
Killeagh (51.940363, -8.026993)	2012	Einstein	5	2nd May	37	23rd May	45	25th June	73
Knockbeg (52.856745, -6.943295)	2011	Cordiale	4	7th April	32	11th May	39	21st June	71
Oak Park (52.863676, -6.914563)	2012	Cordiale	4	4th May	32	6th June	43	28th June	73
Stamullen (53.613615, -6.311924)	2011	Einstein	5	28th April	32	19th May	45	27th June	69

[a] Resistant rating on a scale of 1-9, 1 = susceptible, 9 = resistant (DAFM https://www.agriculture.gov.ie/publications/2013/)

[b] GS Growth stage (Zadoks et al., 1974)

Table 2 Treatments used: application pattern, dose rates applied, fungicides used and actual amount of active ingredient (a.i.) at each treatment time

Application pattern	*Treatment name* [a]	*Dose* [b]	Active ingredient (a.i) applied		*Litres/ha applied at each of T1 & T2 (total a.i. applied)* [d]
			T1 [c]	*T2* [c]	
Un-Treated	Un-T	0	None	None	N/A (0 g)
Solo	EE	1	Epoxiconazole	Epoxiconazole	1.5 (249 g)
	MM	1	Metconazole	Metconazole	1.5 (180 g)
	ee	0.5	Epoxiconazole	Epoxiconazole	0.75 (124.5 g)
	mm	0.5	Metconazole	Metconazole	0.75 (90 g)
Alternation	EM	1	Epoxiconazole	Metconazole	1.5 (214 g)
	ME	1	Metconazole	Epoxiconazole	1.5 (214g)
	em	0.5	Epoxiconazole	Metconazole	0.75 (107 g)
	me	0.5	Metconazole	Epoxiconazole	0.75 (107 g)
Mixture	EMEM	1	Epoxiconazole & metconazole	Epoxiconazole & metconazole	3 (390 g)
	emem	0.5	Epoxiconazole & metconazole	Epoxiconazole & metconazole	1.5 (195 g)

[a] Abbreviations denote the first and second sprays. Un-T= untreated control; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose

[b] Application dose at Treatment 1 and Treatment 2; 1 = the full label recommended dose, 0.5 = half the label recommended dose

[c] Epoxiconazole = Opus Max, Metconazole = Caramba, Epoxiconazole + Metconazole = Gleam. All fungicides are BASF products

[d] Active ingredient (a.i.) per litre of product; Opus max: 83 g/l; Caramba: 60 g/l; Gleam: 37.5 g/l epoxiconazole + 27.5 g/l metconazole

Table 3 Mean sensitivity ($log_{10}EC_{50}$ mg/l) of individual treatments, including pre-treatment, over all sites to (a) epoxiconazole and (b) metconazole, and broken down into treatment means per site

a	Sensitivity to epoxiconazole ($log10EC_{50}$ mg/l)								
	Experiment average			Site					
Treatment[a]	*N*	Mean	*SE*	Duleek (*n*=770)	Julienstown (*n*=449)	Killeagh (*n*=502)	Knockbeg (*n*=710)	Oak Park (*n*=490)	Stamullen (*n*=782)
Pre-T	176	-0.479	0.0711	-0.457	-0.427	-0.480	-0.641	-0.449	-0.403
Un-T	357	-0.377	0.0687	-0.438	-0.355	-0.344	-0.464	-0.330	-0.328
EE	391	-0.042	0.0685	-0.112	0.141	-0.012	-0.135	-0.114	-0.014
MM	388	-0.209	0.0686	-0.151	0.001	-0.292	-0.393	-0.347	-0.079
ee	325	-0.131	0.0691	-0.258	-0.055	0.031	-0.369	-0.218	0.079
mm	356	-0.292	0.0688	-0.274	-0.199	-0.283	-0.536	-0.269	-0.195
EM	379	-0.173	0.0687	-0.235	0.077	-0.368	-0.258	-0.266	0.006
ME	371	-0.054	0.0687	-0.058	0.140	0.038	-0.313	-0.302	0.162
em	168	-0.167	0.0832	-0.191	*	*	-0.404	*	0.030
me	172	-0.111	0.0825	-0.011	*	*	-0.435	*	0.039
EMEM	313	-0.034	0.0694	0.081	0.370	-0.189	-0.342	-0.133	-0.012
emem	307	-0.044	0.0692	0.142	0.217	-0.181	-0.321	-0.054	-0.090

b

	Sensitivity to metconazole (log10EC_{50} mg/l)								
	Experiment average			Site					
Treatment[a]	*N*	Mean[b]	*SE*	Duleek (*n*=762)	Julienstown (*n*=448)	Killeagh (*n*=503)	Knockbeg (*n*=708)	Oak Park (*n*=489)	Stamullen (*n*=773)
Pre-T	176	-0.780	0.0606	-0.893	-0.713	-0.505	-0.957	-0.865	-0.728
Un-T	350	-0.765	0.0583	-0.902	-0.918	-0.837	-0.730	-0.525	-0.678
EE	389	-0.650	0.0581	-0.694	-0.725	-0.672	-0.717	-0.653	-0.449
MM	388	-0.507	0.0582	-0.635	-0.502	-0.568	-0.540	-0.509	-0.293
ee	325	-0.673	0.0586	-0.856	-0.864	-0.679	-0.733	-0.508	-0.406
mm	356	-0.533	0.0583	-0.631	-0.679	-0.385	-0.649	-0.423	-0.437
EM	379	-0.507	0.0582	-0.690	-0.357	-0.595	-0.615	-0.368	-0.406
ME	366	-0.591	0.0584	-0.654	-0.514	-0.565	-0.666	-0.706	-0.444
em	166	-0.641	0.0766	-0.823	*	*	-0.821	*	-0.289
me	170	-0.570	0.0758	-0.609	*	*	-0.734	*	-0.403
EMEM	312	-0.453	0.059	-0.419	-0.187	-0.508	-0.619	-0.614	-0.378
emem	306	-0.505	0.0587	-0.499	-0.390	-0.525	-0.642	-0.529	-0.452

[a] Treatment information in Table 2. Briefly, Pre-T=pre-treatment sample, Un-T= untreated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose

[b] Overall treatment means were calculated using REMLwith site, site.treatment and replicate within site.treatment as random factors

N = total number of isolates from each treatment group.

n = total number of isolates from each site

Table 4 Independent single degree of freedom contrasts between treatments for (a) epoxiconazole and (b) metconazole sensitivity

Contrast	*Contrast sizes*	*P* [a]	Treatment coefficients* included in each contrast question										
			Un-T [b]	EE	MM	ee	mm	EM	ME	em	me	EMEM	emem
Epoxiconazole													
1a. Effect of fungicide	0.253	<0.001	-10	1	1	1	1	1	1	1	1	1	1
2a. Treatments with any epoxiconazole cf. those without	0.155	<0.001	0	1	-4	1	-4	1	1	1	1	1	1
3a. Mixture cf epoxiconazole solo	-0.048	0.3	0	1	0	1	0	0	0	0	0	-1	-1
4a. Treatments with two applications of epoxiconazole cf. those with one	0.064	0.09	0	1	0	1	0	-1	-1	-1	-1	1	1
5a. Order of application of a.i. in alternation	-0.086	0.1	0	0	0	0	0	1	-1	1	-1	0	0
6a. Effect of dose	0.046	0.2	0	1	1	-1	-1	1	1	-1	-1	1	-1
Metconazole													
1b. Effect of fungicide	0.198	<0.001	-10	1	1	1	1	1	1	1	1	1	1
2b. Treatments with any metconazole cf. those without	0.125	0.002	0	-4	1	-4	1	1	1	1	1	1	1
3b. Mixture cf metconazole solo	-0.038	0.4	0	0	1	0	1	0	0	0	0	-1	-1
4b. Treatments with two applications of metconazole cf. those with one	0.084	0.03	0	0	1	0	1	-1	-1	-1	-1	1	1
5b. Order of application of a.i. in alternation	0.006	0.9	0	0	0	0	0	1	-1	1	-1	0	0

6b. Effect of dose	0.044	0.2	0	1	1	-1	-1	1	1	-1	-1	1	-1

[a] *P*-value (in parenthesis) is based on the F-distribution. Error term includes the interaction of treatment with site-year effect. That is, *P*-values allow for variation in the effects of a treatment in different sites –years, and are therefore quite conservative

[b] Treatment information in Table 2. Briefly, Un-T= untreated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose

*Each coefficient denotes the weight by which a mean value was multiplied to calculate the contrast

Table 5 Independent single degree of freedom contrasts between treatments in common azole sensitivity (PC1 in a principal component transformation of the data), and in the difference between epoxiconazole and metconazole sensitivity (PC2 in a principal component transformation of the data)

Contrast	*Contrast sizes*	*P* [a]	Treatment coefficients* included in each contrast question										
			Un-T [b]	EE	MM	ee	mm	EM	ME	em	me	EMEM	emem
PC1													
1a. Effect of fungicide	0.319	<0.001	-10	1	1	1	1	1	1	1	1	1	1
2a. Epoxiconazole solo cf. metconazole solo	0.028	0.6	0	1	-1	1	-1	0	0	0	0	0	0
3a. Mixture cf. solo fungicides	0.171	0.002	0	1	1	1	1	0	0	0	0	-2	-2
4a. Treatments with two applications of a triazole cf. those with one	0.015	0.8	0	1	1	1	1	-1.5	-1.5	-1.5	-1.5	1	1
5a. Order of application of a.i. in alternation	0.07	0.3	0	0	0	0	0	1	-1	1	-1	0	0
6a. Effect of dose	0.066	0.12	0	1	1	-1	-1	1	1	-1	-1	1	-1
PC2													
1b. Effect of fungicide	0.0055	0.9	-10	1	1	1	1	1	1	1	1	1	1
2b. Epoxiconazole solo cf. metconazole solo	0.214	<0.001	0	1	-1	1	-1	0	0	0	0	0	0
3b. Mixture cf. solo fungicides	0.003	0.9	0	1	1	1	1	0	0	0	0	-2	-2
4b. Treatments with two applications of a triazole cf. those with one	-0.025	0.2	0	1	1	1	1	-1.5	-1.5	-1.5	-1.5	1	1

5b. Order of application of a.i. in alternation	0.068	0.05	0	0	0	0	0	1	-1	1	-1	0	0
6b. Effect of dose	-0.002	0.9	0	1	1	-1	-1	1	1	-1	-1	1	-1

[a] *P*-value (in parenthesis) is based on the F-distribution. Error term includes the interaction of treatment with site effect. That is, *P*-values allow for variation in the effects of a treatment in different sites –years, and are therefore quite conservative

[b] Treatment information in Table 2. Briefly, Un-T= untreated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose

*Each coefficient denotes the weight by which a mean value was multiplied to calculate the contrast

Table 6 Differences in yield (t/ha) between treatments at each site, with cross-site analysis

	Site						
Treatment[a]	Duleek	Julienstown	Killeagh	Knockbeg	Oak Park	Stamullen	Mean
Un-T	8.52	4.13	4.82	12.42	5.17	7.32	7.07
EE	10.46	5.68	5.97	14.82	7.19	7.80	8.65
ee	10.00	5.36	4.98	14.83	6.91	7.62	8.28
MM	10.82	5.84	5.70	14.73	7.62	7.44	8.69
mm	10.31	5.28	5.35	13.40	6.74	7.41	8.08
EM	10.62	5.83	4.82	15.32	7.30	7.38	8.54
em	10.27	5.47	6.03	14.30	6.48	7.38	8.32
ME	11.02	5.82	5.24	14.47	7.50	7.55	8.60
me	10.52	5.39	5.44	14.50	6.76	7.21	8.30
EMEM	10.98	6.49	6.41	14.30	8.14	7.27	8.93
emem	10.76	6.03	6.16	14.04	7.42	7.41	8.63
Mean	10.39	5.57	5.54	14.29	7.02	7.44	8.37

	P	*LSD (5% level)*
Site	**<0.001**	0.452
Product[b]	0.17	0.216
Rate[c]	**0.001**	0.193
Site.Product	0.6	0.527
Site.Rate	0.5	0.472
Product.Rate	0.8	0.249
Site.Product.Rate	0.9	0.609

[a] Treatment information in Table 2. Briefly, Un-T= untreated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose

[b] Full and half rates of each treatment (Product) compared; EE+ee, MM+mm, EM+em, ME+me and EMEM+emem

[c] Full rates cf. half rates; EE+MM+EM+ME+EMEM cf. ee+mm+em+me+emem