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Azole and SDHI fungicide sensitivity monitoring of septoria populations (2011–19) and development of tools to rationalise fungicide programmes to control cereal diseases

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1. SUMMARY

1.1 Introduction

Septoria tritici, also known as septoria leaf blotch or septoria tritici blotch (STB), is caused by the fungus *Zymoseptoria tritici* (aka *Mycosphaerella graminicola*). Prevalent since the early 1980s, it is the most important foliar disease of UK wheat (Bearchell *et al*., 2005). The pathogen is also able to develop resistance to foliar fungicides routinely used for its control (Lucas *et al*., 2015). Resistance to methyl benzimidazole carbamate (MBC) and Quinone outside Inhibitor (QoI) fungicides developed rapidly and survey data over the past 20 years shows a gradual erosion of azole efficacy against STB (Gisi *et al*., 2005; Clark, 2006). A key question is whether this erosion is likely to continue or even accelerate with the increased use of azole fungicides to control Septoria. A new generation of succinate dehydrogenase inhibitor (SDHI) fungicides have recently been introduced and are also at risk for resistance development, due to its single site mode of action. A better understanding is needed about the risk of resistance development to SDHIs and further erosion of azole efficacy and what strategies can be undertaken to slow down resistance development to azole and SDHI fungicides.

1.2 Objectives

The aim of the project is to develop tools to assess the risk of resistance development to SDHI fungicides in *Z. tritici* and/or other important UK cereal pathogens. Mutagenesis and subsequent characterisation of SDHI resistant mutants in glasshouse studies will allow us to determine the fitness costs of lab-derived mutants. Using published genome sequences and/or comparative genomics, we will be able to develop high-throughput genotyping tests, based on pyrosequencing, to screen for SDHI-resistance conferring mutations at low frequencies in *Z. tritici* and/or other key UK cereal pathogens. Early detection of resistant alleles at low frequencies, coupled with risk evaluation, will allow the implementation of anti-resistance strategies to prolong the cost-effectiveness and lifetime of this class of fungicides. The project will also monitor the efficacy of azoles and other mixing partners towards different pathogens to support and further rationalise stewardship programmes for SDHI fungicides. The five main research activities to achieve the objectives were:

1. Improve risk assessment of SDHI resistance development in key cereal pathogen populations through fitness cost studies on lab-derived mutants and field isolates.

2. Monitor azole and SDHI sensitivity shifts in *Z. tritici* populations sampled from the AHDB fungicide performance trials and commercial fields.

3. Establish which resistance mechanisms operate in azole and/or SDHI resistant mutants and/or field isolates and identify suitable markers to be used in genotyping tests.

4. Measure the effect of different anti-resistance strategies on the emergence and level of resistance to SDHI fungicides and their mixing partners (e.g. azoles and other active ingredients) in experimental and commercial field trials using high-throughput pheno- and genotyping methods.

5. Devise and disseminate strategies based on appropriate fungicide inputs and sustainable practices to maintain the effectiveness of SDHI based fungicide products.

1.3 Key results

Azole sensitivity shifts are ongoing in Septoria populations with more complex CYP51 variants carrying V136A/C in combination with I381V and S524T spreading and accumulating. These variants show higher levels of insensitivity to all azoles tested, including prothioconazole and epoxiconazole. Lab studies using UV-mutagenised populations under selection pressure by SDHI fungicides showed that resistance can develop rapidly in *Z. tritici*. A range of alterations in succinate dehydrogenase (Sdh) subunits B, C and D were found. These three Sdh subunits form the binding site of SDHI fungicides and mutations were found at some amino acid residues that were predicted from protein modelling and docking studies to interact. Several mutations that were found in field strains since 2015 were identical to some that were found in the lab mutants. One Sdh variant with high levels of insensitivity to all SDHIs tested, C-H152R, shown to have a fitness penalty in lab studies, was also detected in the field but strains carrying this alteration do not seem to accumulate between seasons. In contrast to C-H152R, the most frequently occurring Sdh variants, C-T79N and C-N86S, can be controlled in the glasshouse with a solo SDHI at a quarter rate or less. The diversity of Sdh variants is increasing in Septoria populations and continued monitoring is required to identify potential new Sdh variants with high levels of SDHI insensitivity without carrying a fitness cost.

Results from the field trials show that reduction of the number of azole treatments per crop has the greatest effect on azole resistance build-up. A solo treatment of full label dose azole gave the largest increase in frequency of azole insensitive strains as selection is dose dependent. Increasing doses of SDHI (isopyrazam in this study) as a

mixture partner resulted in decreasing selection for azole insensitive strains (as measured by selection of prochloraz for D134G and/or V136A alleles and against A379G and/or I381V alleles and *vice versa* for tebuconazole selection). Using SDHIs as mixing partner will reduce the selection for insensitivity of the azole fungicides. The larger the SDHI dose in the mixture, the smaller the rate of selection for azole insensitivity. A reduction of the azole dose in the mixture helps reduce selection. For example, the additional benefit of reducing the prochloraz dose for alleviating selection for the V136A mutation was quite small. This follows from the generally weak effect of prochloraz dose on selection seen experimentally. If the opposite is valid for using azoles to protect SDHIs in mixtures then we can expect that mixtures of SDHIs with robust doses of azoles will help increase the effective life of the SDHI fungicides with little deleterious effect on the effective life of the azole component in the mixture (Van den Bosch et al., 2015).

1.4 Conclusions and recommendations for Septoria control

These messages from the project were presented and discussed by the UK Fungicide Resistance Action Group (FRAG) on 26 March 2014. There is good evidence that:

- The shift in azole performance against Septoria continues (HGCA Fungicide Performance data and isolate fungicide sensitivity testing)
- The UK has a more pronounced decline in azole performance than elsewhere in Europe, where disease pressure is lower and azole use is less intense.
- Future azole performance in the UK depends on how we use them.
- The project data shows that selection for azole resistant strains is driven mainly by the number of applications (more sprays = more exposure of the pathogen to the mode of action = more selection).
- Azole use at T0 (for rust) and T3 (for ear blight), in addition to their use at the 'core' T1 and T2 timings, increases selection of insensitive Septoria strains. Any additional sprays will only increase this selection pressure i.e. T1.5 or T4 sprays
- The small incremental shifts in azole sensitivity are only weakly affected by dose.
- Whereas selection for SDHI resistance likely to be increased both by dose and by number of treatments.
- Mixtures reduce selection for resistance.
- Doses of mixture components can be optimised to reduce selection.
- The azole sensitivity situation remains fluid, it is shifted by even single azole applications and the occurrence and frequency of mutations varies from location to location in the UK.

- The project has developed screening methods needed to rapidly screen for the most high-risk SDHI and azole mutations.

The project has provided evidence to support strategies for fungicide usage that reduce the risk of resistance. The dual aim of these strategies is to: 1) Use azoles to protect SDHIs from resistance but minimise selection of azole resistant strains, 2) Use SDHIs to protect azoles from resistance, but minimise selection of SDHI resistant strains. This is a challenging task; to balance the resistance risks against azoles and the SDHIs (the latter remain at high resistance risk) and the need for effective control. The guidance from the SDHI LINK project is to:

- Use the minimum number of azole applications required to achieve effective control. Consider alternatives to azoles where the main target at T0 is Septoria protection (multi-sites are effective in this situation) or rusts (QoIs/stobilurins remain highly effective against rusts). Avoid the use of additional or split sprays (i.e. T1.5, T4).
- The aim is to reduce the number of azole treatments per crop.
- Use mixtures of modes of action effective against Septoria.
- Mix components as follows:
- Maximise control from multi-site acting fungicides (which are at low resistance risk) throughout the programme.
- Use robust doses of azoles at core timings. This will help to achieve effective control, maximise protection of the SDHI components in mixtures and minimise the need for additional /split sprays.
- Use the minimum SDHI dose and number of treatments required to achieve effective control. Partnering with a robust dose of azole will reduce SDHI resistance risk without markedly increasing selection for azole resistance.

The molecular tools developed during these studies, enabling quantitative detection of *Sdh* mutations linked to SDHI insensitivity (SdhC-T79N, C-W80S, C-N86S and C-H152R), are currently used to develop optimal resistance management strategies for Septoria control as part of AHDB project 21120058 'Managing resistance evolving concurrently against two or more modes of action, to extend the effective life of new fungicides': ahdb.org.uk/managing-resistance-evolving-concurrently-against-two-or-more-modes-of-action-to-extend-the-effective-life-of-new-fungicides

With new actives and MOA for septoria control entering the market in 2020, it is crucial to have the most effective and practical resistance management guidance in place from the onset.

2. TECHNICAL REPORT

2.1 Introduction

Septoria tritici blotch (STB), caused by the fungus *Zymoseptoria tritici* is the most important foliar disease of wheat in the UK, and many other countries worldwide. Yield losses due to the disease in the UK have been estimated at around £30M p.a., despite expenditure of £80-90M on fungicides. None of the currently available commercial wheat cultivars are sufficiently resistant to STB to prevent crop losses in seasons of high disease pressure. The primary disease control strategy therefore is, and has been for more than two decades, the programmed application of foliar fungicides to prevent disease spreading to upper leaves, thereby extending green canopy duration (Bertelsen *et al.,* 2001). For the past 30 years target site-specific systemic fungicides, such as the methyl benzimidazole carbamates (MBCs), sterol demethylation inhibitors (DMIs) and strobilurins (QoIs), have dominated the cereal fungicide market because of their efficacy and flexibility regarding timing and delivery. However, *Z. tritici* populations have quickly adapted to the use of such fungicides. Resistance to MBCs, based on a mutation in the beta-tubulin target resulting in replacement of glutamic acid by alanine at codon 198 (E198A), emerged in the mid-1980s (Griffin & Fisher, 1985) and is still prevalent in UK populations of *Z. tritici*, despite the withdrawal of MBCs from spray programmes (Lucas & Fraaije, 2008). More recently, in 2002, resistance to strobilurin fungicides (correlated with a mutation in the mitochondrial cytochrome *b* target resulting in the replacement of glycine by alanine at codon 143 (G143A)) was detected in *Z. tritici* populations in the UK and Ireland (Fraaije *et al*., 2003). By spring 2003, *M. graminicola* isolates with the G143A mutation were widespread and common in the UK, accounting for around 35% of the pathogen population, increasing by the end of the season to 80-90%, despite measures implemented to reduce selection for resistance (Fraaije *et al*., 2005a). QoI fungicides can therefore no longer be relied upon to provide adequate control, and fungicide programmes are now based on the use of the azole group of DMIs, a new generation of carboxamide fungicides inhibiting succinate dehydrogenase (Sdh) of the mitochondrial respiratory chain (complex II) and multi-site inhibitors.

During the 1990s, monitoring studies suggested that only slight shifts in sensitivity to azoles had occurred, and these were not sufficient to compromise control of STB (Hollomon *et al*., 2002). Resistance development to sterol DeMethylation Inhibitor (DMI) has developed slowly, in a stepwise manner and can be compound dependent (Fraaije et al., 2007). However, comparisons of field performance of azoles over the

past decade has shown a clear erosion of efficacy, so that higher doses of azoles are now required to achieve the same level of disease control (see Clark, 2006). Variation in base-line sensitivity to azoles is a polygenic trait, in which several different mechanisms can contribute to resistance (Stergiopoulos *et al.*, 2003). Studies with isolates collected over time have demonstrated that reduced sensitivities are mainly associated with multiple alterations in the sterol 14α-demethylase target protein (CYP51) (e.g. mutations leading to amino acid substitutions and deletion of two amino acids) (see Figure 1) (Cools & Fraaije, 2008; Cools *et al.*, 2012).

Figure 1. CYP51 amino acid sequence alignment and positions of alterations in *Zymoseptoria tritici*. Z.t. (*Z. tritici*), C. a. (*Candida albicans*) and M. t. (*Mycobacterium tuberculosis*). Arrows in red display alterations known or predicted to impact direct on azole binding (D134G, V136A/C/G, Y137F, H303Y, A311G, G312A, A379G, I381V, ΔY459/G460, Y459D, G460D, Y461H/S, F490L and S524T). Alterations in green are not directly involved in azole binding (L50S, D107V, S188N and N513K). The different conserved substrate recognition site areas are marked blue.
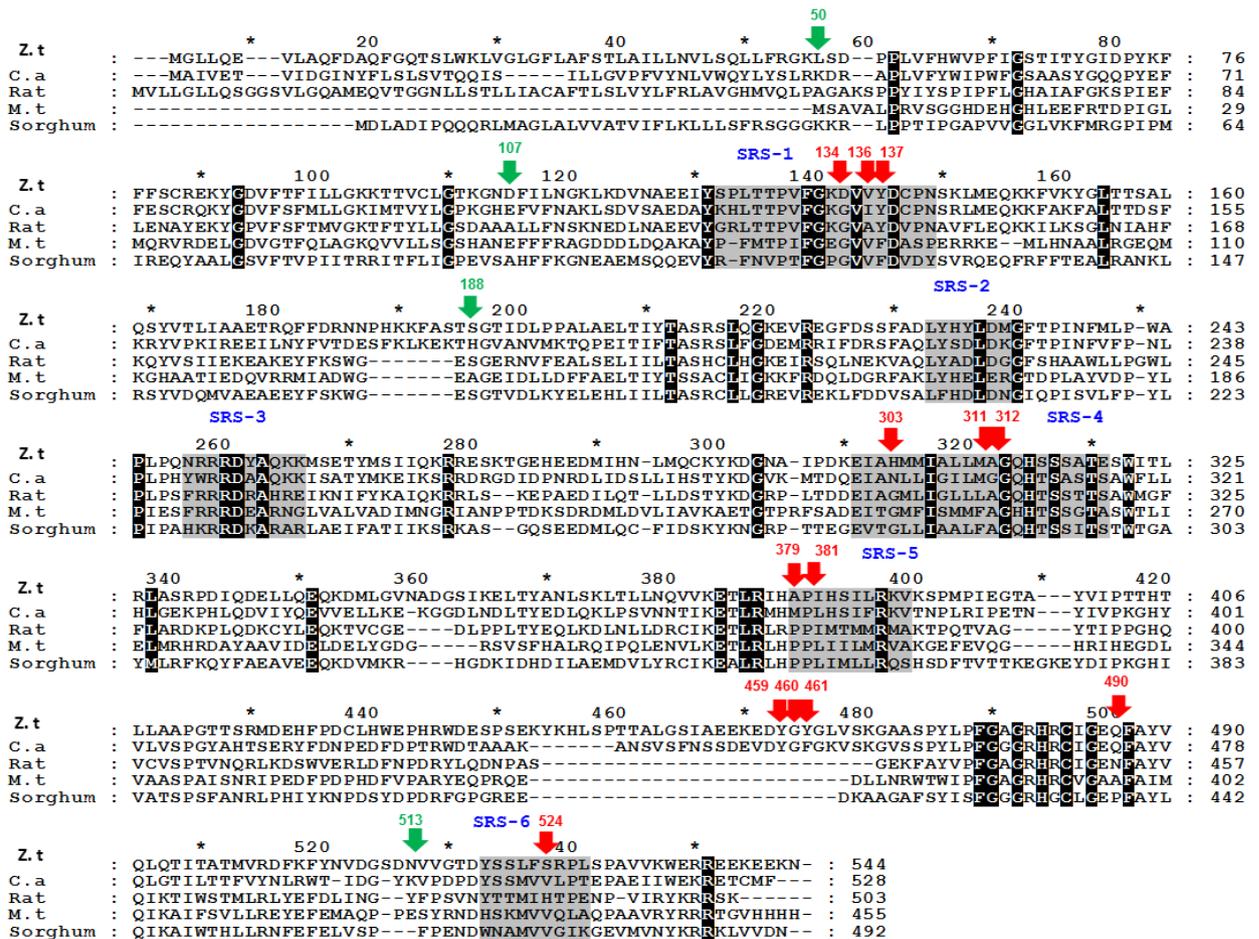
More recently, *CYP51* overexpression (Cools *et al*., 2012) and changes in the expression of efflux proteins that transport toxins out of the cell (Leroux & Walker, 2011; Omrane *et al*., 2015) have also been implicated to contribute to resistance development (Cools & Fraaije, 2013). Despite a gradual erosion of azole efficacy against STB (see Gisi et al., 2005; Clark, 2006), the most active azoles, epoxiconazole and prothioconazole, still provide good disease control at robust rates when used in a protectant situation but a key question now is whether this erosion is likely to continue.

In addition to boscalid, which was launched in 2005, several new generation carboxamide fungicides have entered the UK cereal market during the course of the studies (e.g. isopyrazam (2010), bixafen (2012), fluopyram (2012), fluxapyroxad (2012), penthiopyrad (2014), benzovindiflupyr (2017) and sedaxane (2017) (see https://secure.fera.defra.gov.uk/pusstats/). In contrast to early generation Sdh inhibitors (SDHIs), for example carboxin, these new compounds have a wide spectrum of activity and some curative properties. Due to their single site mode of action, the risk of resistance developing to SDHIs is high. Up till 2010, at the start of our studies, resistance to SDHI fungicides was not detected in field isolates of important UK cereal pathogens including *Z. tritici, Puccinia recondita, Puccinia hordei, Oculimacula herpotrichoides, Pyrenophora teres*, *Ramularia collo-cygni* and *Rhynchosporium commune*. However, carboxin-resistant lab mutants of *Z. tritici* were generated at Long Ashton research station in 1998 and are cross-resistant to boscalid (Skinner *et al*., 1998). These mutants carry amino acid substitutions H267L and H267Y in the Sdh iron sulfur protein subunit (SdhB). Recently, similar or identical mutations at this codon and mutations at other positions within SdhB, and subunits SdhC and SdhD have also been found to confer resistance to SDHI fungicides in field isolates of *Botrytis cinerea* (strawberry)*, Botrytis elliptica* (lily), *Alternaria alternata* (pistachio)*, Corynespora cassiicola* (cucurbits)*, Didymella bryoniae* (water melon) and *Podosphaera xanthii* (cucurbits) (Sierotzki & Scalliet, 2013)*.

Given current uncertainties about sustainability of chemical control of crop diseases, due to new legislation for registration of crop protection products (Regulation EC 1107/2009) and resistance development, good stewardship of this new chemistry is essential. To prevent or delay resistance development to SDHI fungicides in cereal pathogens, the Fungicide Resistance Action Committee (FRAC) has recommended using SDHI fungicides preventatively in mixtures with a maximum of two sprays per season. Mixing partners should provide satisfactory disease control when used alone and have a different mode of action. Unfortunately, although multi-

site inhibitors can also be considered, azoles are the most suitable mixing partner for SDHI applications for control of STB in the UK due to their greater efficacy, curative properties and/or wider spectrum of disease control.

Objectives

A main goal of the studies was to develop tools to assess the risk of resistance development to SDHI fungicides in *Z. tritici* and/or other important UK cereal pathogens. Mutagenesis and subsequent characterisation of SDHI resistant mutants in glasshouse studies will allow us to determine the fitness costs of lab-derived mutants. Using published genome sequences and/or comparative genomics, we will be able to develop high-throughput genotyping tests based on pyrosequencing to screen for SDHI-resistance conferring mutations at low frequencies in *Z. tritici* and/or other key UK cereal pathogens. Early detection of resistant alleles at low frequencies, coupled with risk evaluation, will allow the implementation of anti-resistance strategies to prolong the cost-effectiveness and lifetime of this class of fungicides. The project will also monitor the efficacy of azoles and other mixing partners towards different pathogens to support and further rationalise stewardship programmes for SDHI fungicides.

The five main research activities to achieve the objectives were:
1. Improve risk assessment of SDHI resistance development in key cereal pathogen populations through fitness cost studies on lab-derived mutants and field isolates.
2. Monitor azole and SDHI sensitivity shifts in *Z. tritici* populations sampled from the AHDB Fungicide Performance trials and commercial fields.
3. Establish which resistance mechanisms operate in azole and/or SDHI resistant mutants and/or field isolates and identify suitable markers to be used in genotyping tests.
4. Measure the effect of different anti-resistance strategies on the emergence and level of resistance to SDHI fungicides and their mixing partners (e.g. azoles and other active ingredients) in experimental and commercial field trials using high-throughput pheno- and genotyping methods.
5. Devise and disseminate strategies based on appropriate fungicide inputs and sustainable practices to maintain the effectiveness of SDHI based fungicide products.

2.2 Materials and methods

2.2.1 *In silico* studies on the binding of SDHIs to the Sdh complex of *Zymoseptoria tritici*

For docking studies of fungicides in the *Z. tritici* Sdh complex, the crystallographic coordinates of the avian Sdh complex was taken from the Protein Data Bank (PDB). The Sdh complex of chicken (PDB code 2FBW) is similar to that of *Z. tritici* (70% sequence identity) and contains a bound carboxin molecule. By replacing all residues within a 14 Å radius of the bound carboxin with the corresponding residues of the *Z. tritici* Sdh complex by using PyMOL, a structural model of the fungal quinone binding site was prepared. All fungicides tested were also prepared in PyMOL. The Molegro Virtual Docker (Molegro ApS, Aarhus, Denmark) was used for docking simulations. For docking simulations, the structural model and all fungicides were first subjected to energy minimization in MVD. For each resistance-related mutation in one of the subunits, the corresponding mutant protein was built and optimized. Fungicides were docked within a sphere (radius of 12 Å) that encompassed the quinone binding site. For docking, the default settings were used except for the grid resolution, which was set to 0.2, and a total of 25 runs were performed with a maximum of 500 iterations. The best superimposed positions with lowest MolDock energy scores were used in this study. For visualization of the docked fungicides PyMOL was used.

2.2.2 Isolation of *Zymoseptoria tritici* strains

After sampling leaves from field trials, leaves or leaf segments containing lesions with pycnidia were washed and stapled onto round filter paper. Filters were placed in Petri dishes and wetted without an excess of water. After 24 h incubation at 20°C in the dark, oozing cirri from individual pycnidia were detached with pointed watchmaker forceps under a microscope and suspended in 30 μl of sterile water. A loopful of spore suspension was plated out on yeast extract peptone dextrose agar (YEPD; ForMedium, Norwich, UK) amended with 100 μg ml^{-1} of penicillin G and streptomycin sulphate and incubated for 5 days in the dark at 20°C. To obtain single spore cultures, isolates from single colonies were sub-cultured twice on agar. Spore suspensions of isolates were either used directly in fungicide sensitivity assays after counting spore numbers under a microscope or stored in 50 % (v/v) glycerol at -80°C.

2.2.3 Generation of SDHI-resistant UV-mutants

Ten thousand spores of *Z. tritici* strains IPO323 or IRE30 were plated out on YEPD agar amended with carboxin at 50 µg mL^{-1}. Plates were exposed to UV light at 500 or 600 J m^2 using a Stratagene UV-crosslinker (model 1800) which resulted in approximately 20 % survival. Putative carboxin-resistant mutants were isolated from growing colonies after 14 days incubation at 21 °C in the dark. These mutants were grown for another generation on carboxin-amended YEPD agar, harvested in sterile distilled water and stored in 80% (v/v) glycerol at -80°C for further use.

2.2.4 *In vitro* fungicide sensitivity testing of *Zymoseptoria tritici* field strains and mutants

In vitro sensitivity assays were carried out according to Pijls *et al*. (1994) with the following modifications. Wells of flat-bottomed microtitre plates (Greiner, product 92696) were filled with 100 µl of double strength Sabouraud liquid medium (modified) (SDLM; Oxoid, Basingstoke, UK) amended with 11 different concentrations of bixafen, boscalid, carboxin, isopyrazam, epoxiconazole, prothioconazole-desthio, a metabolite of prothioconazole (Parker *et al*., 2011), and chlorothalonil. Before dilution in media, chemicals were dissolved in acetone with exception of chlorothalonil which was dissolved in DMSO. One hundred µl of spore suspensions (approximately 2500 spores) of *Z. tritici* strains or UV mutants were added to each well. Plates were incubated for four days at 23°C, and growth measured at 630 nm using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany), in well-scanning mode with a 2 by 2 matrix of scanning points within a 3 mm diameter. Fungicide sensitivities were determined as 50% effective concentration (EC$_{50}$ in µg/mL) using a dose-response relationship (4-parameter fit) according to the OPTIMA Software.

2.2.5 *In planta* fitness studies on Sdh mutants and fungicide sensitivity testing of *Zymoseptoria tritici* strains field strains

In planta pathogenicity tests were carried out on 2 to 3-weeks old wheat seedlings of cultivar Riband according to Keon *et al*. (2007). The second leaf of each wheat plant was attached, adaxial side up, to perspex sheet frames and 4 cm of leaf inoculated with spore suspensions (approximately 2.5x10^5 spores ml^{-1} in 0.1 % (v/v) Tween-20) using a mini 'mist' sprayer (inoculation of approximately 100 µl of inoculum per leaf segment). In total, 18 leaves per strain were inoculated. After 72 h incubation at

100% relative humidity in polystyrene boxes, inoculated plants were incubated at 16 °C with a 16 h light period at 88 % relative humidity for up to 25 days to allow symptoms to develop. Visual assessments, recording the extent of pycnidia formation and chlorosis, were conducted after 21 days. DNA was also extracted from four 2-cm inoculated leaf segments at several time points (7, 14, 21 and 25 days after inoculation) and amount of pathogen DNA quantified in 50 ng DNA samples according to Fraaije *et al*. (2005) using the CY5 probe to quantify the presence of the cytochrome *b* gene of *Z. tritici*. Spores were also counted from three inoculated leaves after 21 days. Three 2-cm leaf segments were added to 2 ml tubes and incubated overnight in the dark at room temperature in the presence of 300 µl of 0.2 % Tween-20 solution. Spores were harvested after two rounds of spinning and shaking at 4.0 m/s in a FastPrep machine (Savants Instruments, Holbrook, USA). Amounts of spores collected were determined microscopically using a haemocytometer.

   For fungicide sensitivity testing, fungicides were applied as 1-day preventative sprays using a hand-held sprayer until runoff. After spraying fungicides (Folicur (a.i. tebuconazole) at 1.5, 4.4, 13, 40, 120 and 360 ppm; Opus (epoxiconazole) at 0.3, 0.7, 2.2, 6.7, 20, 60 ppm; Poraz (prochloraz) at 0.8, 2.5, 7.4, 22, 67 and 200 ppm; Proline (prothioconazole) at 0.74, 2.2, 6.7, 20, 60 and 180 ppm) in the presence of 0.1 % (v/v) of Tween-20 (wetting agent), leaves were allowed to dry overnight in a growth room before inoculation with spores as described for the Sdh mutants. For each strain, three leaves per fungicide concentration were tested and visual assessments, recording the extent of pycnidia formation and chlorosis, were conducted to establish the efficacy of the different fungicide sprays after 21 days.

2.2.6 DNA extractions and quantification

DNA was extracted directly from mycelium or leaves by powdering samples in liquid nitrogen using a pestle and a mortar or, alternatively, crushing the leaves with a Pohlähne roller press whilst adding DNA extraction buffer. To each powdered sample, DNA extraction buffer consisting of 40 µl 1% (v/v) β-mercapthoethanol, 400 µl TEN buffer (500 mM NaCl, 400 mM Tris-HCl, 50 mM EDTA, 5 mM 1,10-phenanthroline monohydrate, 2 % (w/v) polyvinylpyrrolidone; pH 8.0) and 400 µl 2% (w/v) SDS was added. For larger wheat leaf samples, the amount of DNA extraction buffer added was increased until the mixture could be poured. After incubating the mixture for 30 min at 70°C, 400 µl ice-cold ammonium acetate (7.5 M) was mixed with the heat-treated sample and the total suspension kept on ice for 30 min. After centrifugation at 10,000 rpm for 10 min, an equal volume of cold (-20°C) isopropanol was added to the

supernatant and the extract shaken at room temperature for 15 min. After centrifugation at 6,000 rpm for 5 min, DNA pellets were washed with ice-cold 70% (v/v) ethanol, centrifuged again and dissolved in 500 µl sterile distilled water. Thiazole orange is a cyanine dye that specifically binds to double-stranded DNA and can be used for fluorimetric detection and quantification of DNA. From each crude DNA extract and calibration sample with a known amount of DNA, 2 µl of sample was incubated at room temperature in a microtitre plate well with 150 µl of a 2.5 µM thiazole orange solution (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Fifteen min after excitation at 480 nm, fluorescence was measured at 530 nm using a FLx800 fluorimeter (Bio-Tek Instruments Inc., Winooski, VT) and DNA concentrations calculated using appropriate calibration curves generated from calibration sample measurements.

2.2.7 Cloning and sequencing of genes encoding CYP51 and Sdh subunits B, C and D

A large fragment of the *CYP51* gene of *Z. tritici* was amplified with primer set CYP51F1 (5'-TTCTCCCGGAACATTGACAT-3')/CYP51R1 (5'-TGCATACCCACACCAATTCT-3'). PCR was carried out in 25 µl reactions, consisting of 2 µl of DNA sample (20 ng of genomic DNA), 5 µl of 5X Phusion HF buffer, 0.125 µl of each primer (100 µM), 0.5 µl of dNTP solution (10 mM of each dNTP), 0.25 µl DNA polymerase (2 U µl^{-1}) and 17 µl of sterile distilled water, using a Biometra T3 thermocycler (Biotron GmbH, Göttingen, Germany) with Phusion Hot Start High-Fidelity DNA polymerase (Finnzymes) under the following conditions: initial denaturation at 98°C for 30 s, followed by 40 cycles of 98°C for 5 s, 60°C for 20 s, 72°C for 1 minute with a final extension at 72°C for 5 minutes. Presence of PCR products was confirmed on ethidium bromide-stained 1.3 % (w/v) agarose gels run in 1X Tris-borate-EDTA buffer and exposed to UV light to visualise DNA fragments. PCR products were directly sequenced with the PCR primers and CYP51S3 (5'-AGAAGTTCGCATCGAC–3') using a dideoxy chain termination method reaction. Sequences were assembled and further aligned with Vector NTI and Clustal Software.

The genes encoding for the Sdh B and D subunits were also amplified with with Phusion Hot Start High-Fidelity DNA polymerase using primer sets SDHBF1 (5'-TAACACTCCACGCCTCACG-3') /SDHBR1 (5'-GTCTTCCGTCGATTTCGAGAC-3') and SDHDF1 (5'-GGCATCATCGTCAAGCAAG-3')/SDHDR1 (5'-CAATTCTTCTTGGCAGCAACA-3'), respectively. With exception of a primer annealing temperature of 63°C, the PCR conditions were identical to that of the *CYP51* amplification. The gene encoding Sdh C

was amplified with Easy A High-Fidelity Cloning Enzyme, a proprietary thermostable DNA polymerase formulation (Agilent Technologies), using primer set SDHCF1 (5'-TCCTGTCCTGTGATCCTGGA-3') /SDHCR1 (5'-TCCCTTGGGTCCTGATGTAC-3'). PCR was carried out in 25 μl reactions, consisting of 2 μl of DNA sample (20 ng of genomic DNA), 2.5 μl of 10X Easy-A reaction buffer, 0.125 μl of each primer (100 μM), 0.5 μl of dNTP solution (10 mM of each dNTP), 0.25 μl Easy A High-Fidelity Cloning Enzyme (5 U μl^{-1}) and 19.5 μl of sterile distilled water. Reaction conditions on the Biometra thermal cycler were: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 s, 63°C for 20 s, 72°C for 2 minutes with a final extension at 72°C for 10 minutes.

2.2.8 Pyrosequencing assays for detection of mutations in SdhC and CYP51 encoding genes in *Zymoseptoria tritici* isolates and field samples

A nested PCR approach was carried out for all Pyrosequencing assays. To detect and quantify mutations leading to CYP51 D134G and V136A simultaneously, the first round PCR reaction carried out with Red Hot DNA polymerase (ABgene, Epsom, UK) used primer set ST51F1 (3'-ATGGGTCTCCTCCAGGAAGTCCTC-5')/BBR3 (3'-CGTACTTGACGAACTAAGTGACAAGTC-5'), generating a 517 bp PCR product covering CYP51 codons 9-149. For simultaneous detection and quantification of A379G and I381V, primer set F2BF (3'-CCATGACATCGCCGAGCATTTGC-5')/BF381R2 (3'-GCTCTCGTCCCATCGATGCGGCTC-5') was used, generating a 616 bp PCR product covering CYP51 codons 280-427. Primers ZTCF1 (3'-AACGAAATCCTCGCCAAACA-5') and ZTCR1 (3'-CGCAACACTCAACCCCACA-5') were used to generate a 375 bp PCR product covering SdhC codons 55-179. Twenty μl reactions were carried out, consisting of 2.5 μl of DNA sample (50 ng of DNA), 2 μl of 10X Red Hot Taq buffer, 0.1 μl of each primer (100 μM), 0.4 μl of dNTP solution (10 mM of each dNTP), 1.2 μl magnesium chloride (25 mM), 0.04 μl Red Hot DNA polymerase (5 U μl^{-1}) and 13.66 μl of sterile distilled water. PCR was carried out in a Biometra T3 thermocycler under the following conditions: initial denaturation at 94°C for 2 minutes, followed by 40 cycles of 94°C for 10 s, 63°C (ST51F1/BBR3) or 65°C (F2BF/BF381R2 and ZTCF1/ZTCR1) for 30 s, 72°C for 45 s with a final extension at 72°C for 5 minutes.

For the second round PCR reactions, primers (forward primer, reverse primer and sequence primer) were designed with Pyrosequencing Assay Design Software (Version 1.0.6; Biotage) taking also into account sequence diversity at other nucleotide positions by using degenerate primers. Either the forward or reverse primer was designed with a biotin label at the 5'-end (see Table 1).

Table 1. Pyrosequencing targets, primers and assay nucleotide dispensation order

Target	Oligonucleotide sets[1]	Sequence to analyse	Nucleotide dispensation order
CYP51			
D134G, V136A	F: ATGGAAAACTGAAGGACGTCAA R: ATGAGCTTCGAATTGGGACAA* S: CCTGTCTTTGGCAAG	GA/GTGTGGT/CTTATGATTGTCCCAATTCG	CGACTGTGTCTGA
A379G, I381V	F: GTCGTCAAAGAAACCCTTCGTATT* R: GACGTATGCCGTACCTTCGAT S: TGCGCAGAATGGAGT	GGAC/TTGGAC/GCGTGAATACGAAGGGTTTCTTTGAC	CGACTCGAGCTG
SdhC			
T79N, W80S	F: CCTCGCAATCTACAAACCGG R: CCGAAGGCGTAGAAGGCT* S: TCTACAAACCGCAAATAA	A/CCTG/CGTACCTC/GTCG/CGC	GACATCGCTACTCGATCG
N86S	F: CCTCGCAATCTACAAACCG R: CCGAAGGCGTAGAAGGCT S: ACCTSTCSGCYCTCA	G/ACCGCGTGACCGG	TGATCGCGT
H152R	F: CGGTGACGTTTCATTCGTT R: GTCTGCACCTGCTTATTCGTAATC* S: TGAATGGAGTGASKC	A/GTTTGGTT/GTGGGATACGGCGAGTATG	CAGCTGTGTGCATA

[1] F, R and S indicate forward, reverse and sequence primers, respectively; Primers with 5' biotin label are marked with an asterisk

PCR reactions were carried out with the One Taq DNA polymerase kit (New England Biolabs) in 40 µl reaction volumes, consisting of 2 µl of DNA sample (first round PCR product 1:500 diluted), 8 µl of 5X One *Taq* standard buffer, 0.2 µl of each primer (100 µM), 0.8 µl of dNTP solution (10 mM of each dNTP), 0.2 µl One *Taq* DNA polymerase (5 U µl^{-1}) and 28.6 µl of sterile distilled water. PCR reactions were run in a Biometra T3 thermocycler under the following conditions: initial denaturation at 94°C for 30 s, followed by 40 cycles of 94°C for 10 s, 54°C for 20 s, 68°C for 30 s with a final extension at 68°C for 4 minutes and 30 s.

Presence of first and second round PCR products was confirmed on ethidium bromide-stained 1.3 % (w/v) agarose gels run in 1X Tris-borate-EDTA buffer and exposed to UV light to visualise DNA fragments. The amplicon sizes of the PCR products were: 103 bp (CYP51 D134G + V136A assay), 52 bp (CYP51 A379G + I381V), 93 bp (SdhC T79N + W80S), 93 bp (SdhC N86S) and 82 bp (SdhC H152R). Single-stranded biotinylated PCR products were prepared for sequencing using the Pyrosequencing Vacuum Prep Tool (Biotage). Three µl Streptavidin Sepharose HP beads (Amersham Biosciences) were added to 40 µl binding buffer (10 mM Tris-HCl pH 7.6, 2 M NaCl, 1 mM EDTA, 0.1 % Tween 20) and mixed with 20 µl PCR product

and 20 µl of sterile distilled water for 10 minutes at room temperature using an Orbis plate shaker (Mikura). Beads containing the captured templates were aspirated onto filters after applying the vacuum, washed with 70% (v/v) ethanol for 5 s, rendered single-stranded with denaturation solution (0.2 M NaOH) for 10 s and neutralised with washing buffer (10 mM Tris-Acetate, pH 7.6) for 5 s. The vacuum pressure was released, and beads transferred into a PSQ 96-well plate (Biotage) containing 45 µl annealing buffer (20 mM Tris-Acetate, 2 mM Mg Acetate, pH 7.6) and 0.5 mM sequence primer. Pyrosequencing reactions were performed according the manufacturer's instructions using the PSQ 96 SNP Reagent kit (Biotage). Assays were performed on the PSQ MA96 (Biotage) using the nucleotide dispensation orders shown in Table 1. The allele frequencies were determined using the PyroMark ID SNP run software. Possible outcomes of the Pyrograms for homogeneous (100 %) and heterogeneous populations (50 % of each allele) of *Z. tritici strains* carrying D134G (gat into ggt) and/or V136A (gtt into gct), and, A379G (gct into ggt) and/or I381V (atc into gtc) are shown in Figures 2 and 3, respectively. Pyrograms for simultaneous detection of SdhC T79N and/or C-W80S in homogeneous and heterogeneous *Z. tritici* populations are shown in Figure 4. Pyrograms for detection of C-N86S and C-H152R in homogeneous and heterogeneous *Z. tritici* populations are presented in Figures 5 and 6, respectively.

Figure 2. Selected theoretical outcomes for simultaneous detection of D134G and V136A in *Zymoseptoria tritici* field populations based on a homogeneous or heterogeneous (50% mixtures) presence of mutations in test samples. Analysed sequence: GA/GTGTGGT/CTTATGATTGTCCCAATTCG.
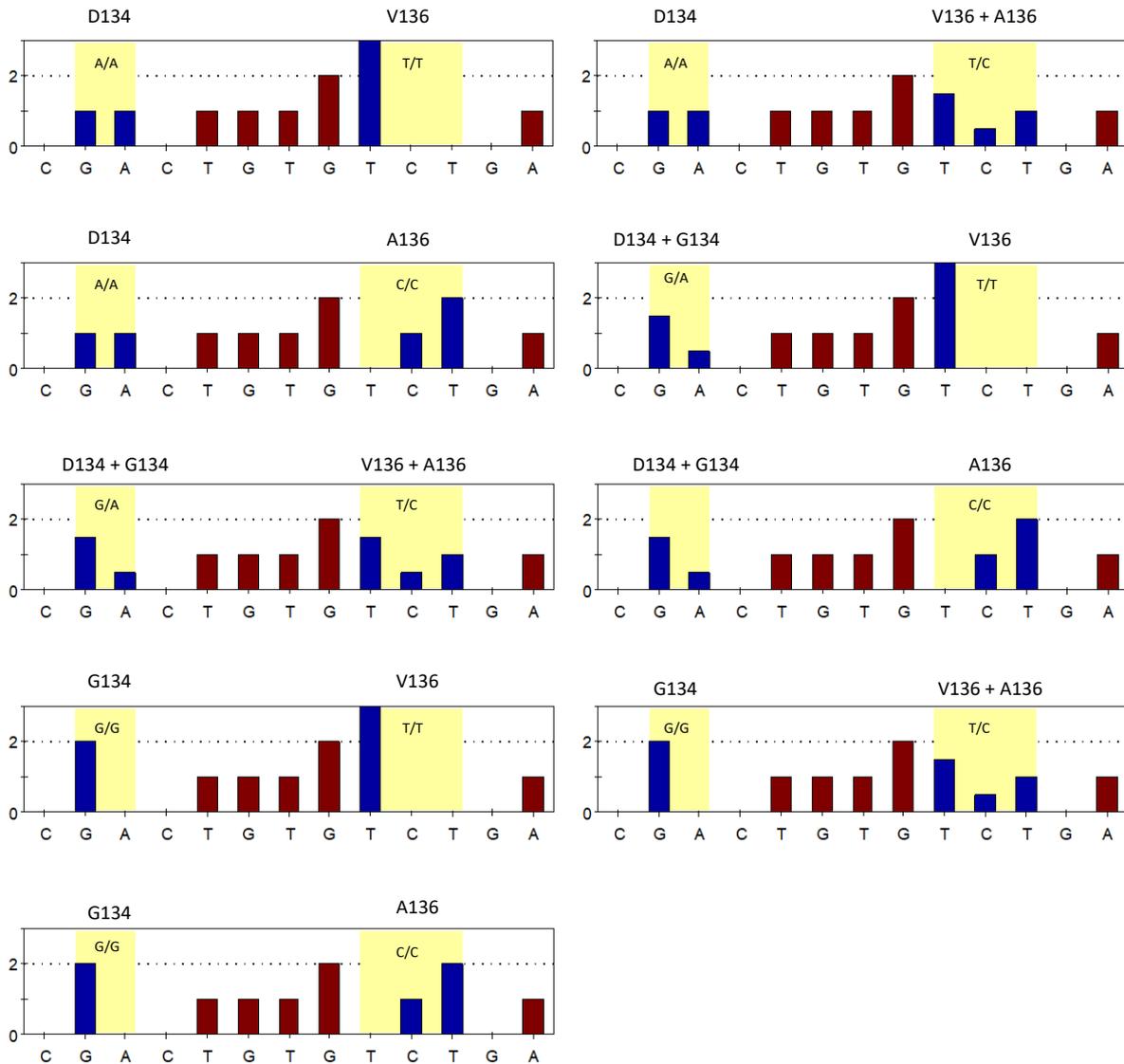
Figure 3. Selected theoretical outcomes for simultaneous detection of A379G (gct to ggt) and I381V (atc to gtc) in *Zymoseptoria tritici* field populations based on a homogeneous or heterogeneous (50% mixtures) presence of mutations in test samples. Sequence (reverse strand was used for SNP detection pyrosequencing assay design) to analyse is: GGAC/TTGGAC/GCGTGAATACGAAGGGTTTCTTTGAC
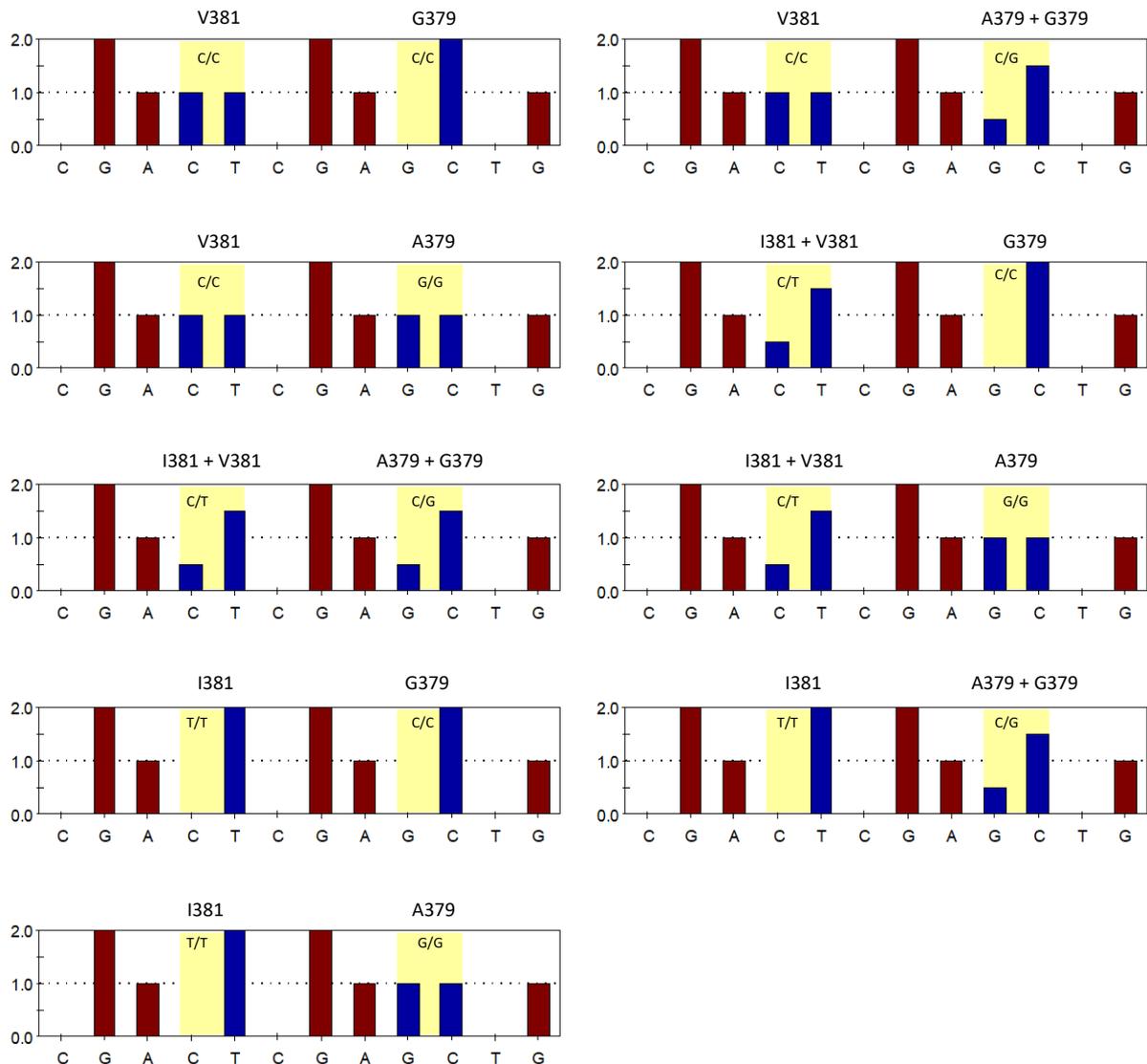
Figure 4. Selected theoretical outcomes for simultaneous detection of SdhC-T79N (acc to a<u>a</u>c) and C-W80S (tgg to t<u>c</u>g) in *Zymoseptoria tritici* field populations based on a homogeneous or heterogeneous (50% mixtures) presence of mutations in test samples. Sequence (reverse strand used for design of the SNP detection pyrosequencing assay) to analyse is: A/CCTG/CGTACCTC/GTCG/CGC
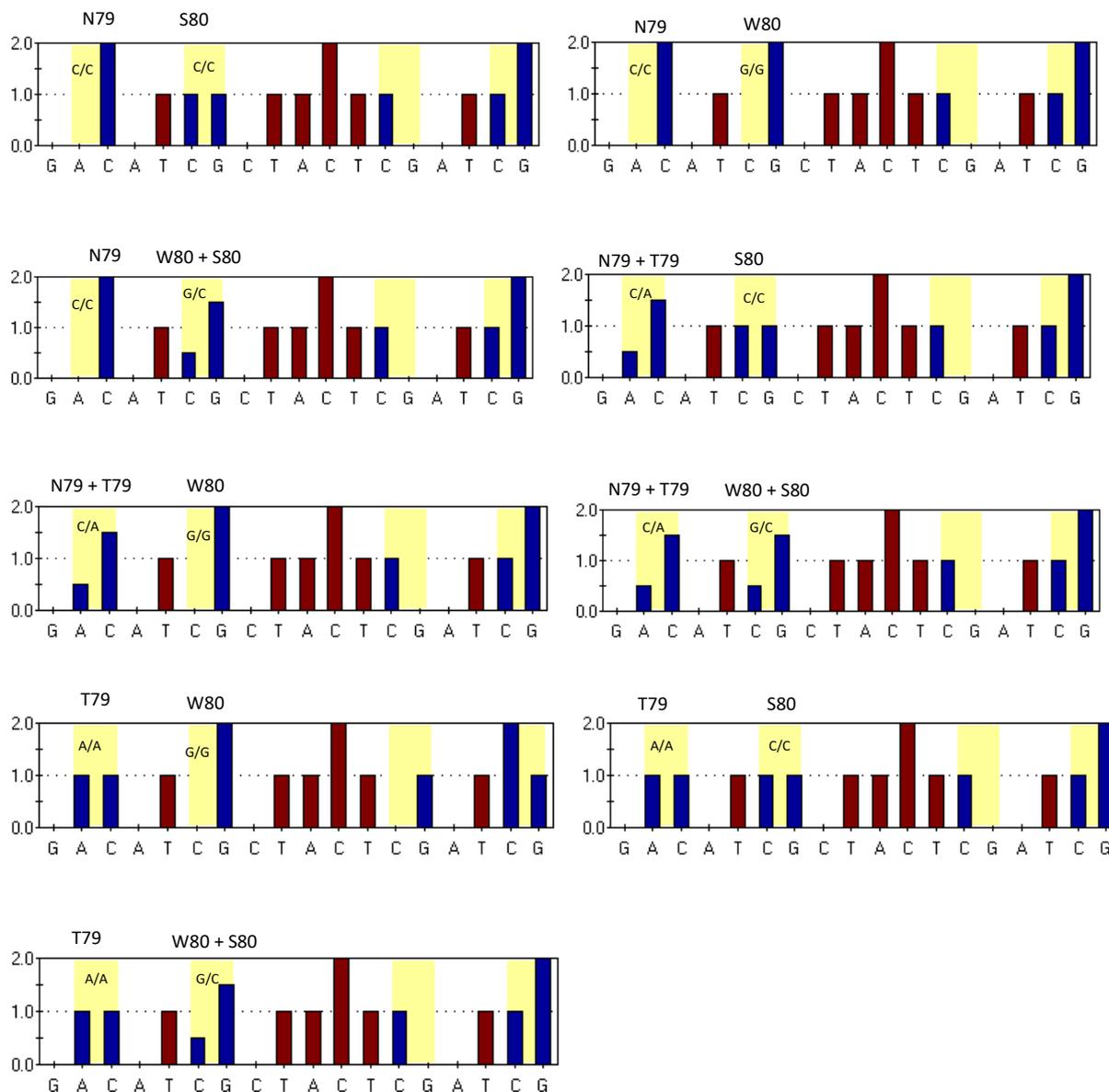
Figure 5. Selected theoretical outcomes for simultaneous detection of SdhC-N86S (aac to a<u>g</u>c) in *Zymoseptoria tritici* field populations based on a homogeneous or heterogeneous (50% mixtures) presence of mutations in test samples. Sequence (reverse strand used for design of the SNP detection pyrosequencing assay) to analyse is: G/ACCGCGTGACCGG
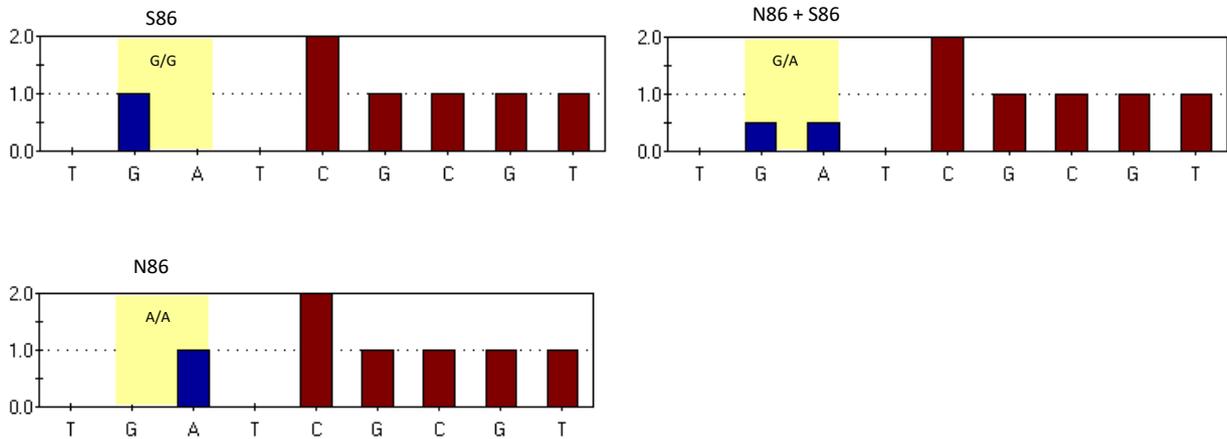
Figure 6. Selected theoretical outcomes for simultaneous detection of SdhC-H152R (cat to cgt) in *Zymoseptoria tritici* field populations based on a homogeneous or heterogeneous (50% mixtures) presence of mutations in test samples. Sequence (reverse strand used for design of the SNP detection pyrosequencing assay) to analyse is: A/GTTTGGTT/GTGGGATACGGCGAGTATG
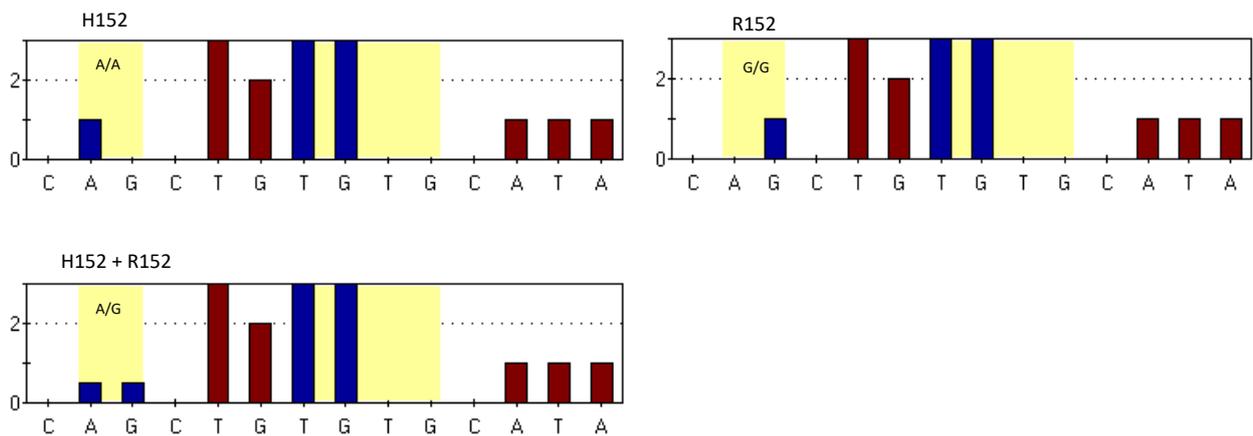
2.3 Core field experiments

Table 2 gives an overview of all active ingredients present in the formulated products used in all core field trials conducted during 2010-2013.

Table 2. Formulated products and active ingredients used in field experiments

Product	Active ingredient	Maximum individual dose (L ha^{-1})	Maximum total dose (L ha^{-1})
Folicur 250	Tebuconazole 250 g L^{-1}	1.0	2.0
Isopyrazam[1]	Isopyrazam 125 g L^{-1}	-	-
Opus 125	Epoxiconazole 125 g L^{-1}	1.0	2.0
Poraz 450	Prochloraz 450 g L^{-1}	0.9	1.8

[1] Product kindly provided by Syngenta. The solo product was initially not registered/commercially available in the UK; isopyrazam was introduced as a mixture with epoxiconazole (Seguris containing epoxiconazole (90 g L^{-1}) to control Septoria leaf blotch in the UK

2.3.1 Design of core field trials 2011-14

Due to absence of SDHI resistance in the field and, as a consequence, lack of DNA based markers to measure the dynamics of fungicide insensitive strains within populations, it was decided to study the role of SDHIs in protecting azole efficacy (reducing azole resistance development) until SDHI resistance was found in field populations of *Z. tritici* or in other key cereal pathogens. Using isopyrazam as mixing partner with different azoles, the effect of dose rate and number of sprays of each mixing partner on selection of azole resistance was investigated. Based on the available phenotyping data of most commonly occurring CYP51 variants (Table 3), it was decided to use mutations linked with CYP51 amino acid substitutions V136A and I381V as key markers. During assay development, it became clear that frequencies of D134G and A379G can be determined simultaneously in the assays developed for V136A and I381V, respectively. Most, if not all, recently evolved CYP51 variants do carry either V136A and/or I381V. Variants carrying both V136A and I381V seem always to have D134G as well. Variants carrying I381V are generally less sensitive to epoxiconazole and tebuconazole, whereas variants with V136A are least sensitive to prochloraz and more sensitive to tebuconazole. A379G is mainly found in one CYP51 variant, which carries I381V as well, and is highly sensitive to prochloraz. All treatments for the core field trials carried out during the growing seasons of 2010/11, 2011/12 and 2012/13 are listed in Tables 4, 5 and 6, respectively.

Treatments were partly designed to reduce the initial frequency of V136A by tebuconazole pre-treatment, then test for the effects of azole/SDHI mixtures, varying dose rates of each partner in balanced mixtures, and alternation on selection for D134G, V136A, A379G and I381V by prochloraz, tebuconazole and epoxiconazole. Additional treatments based on a one or two spray programme were included to obtain response data for prochloraz and isopyrazam. Based on fungicide resistance modelling as described by Van den Bosch *et al*. (2015), the following hypotheses were tested: 1) mixture benefit does not depend on reducing the dose of the azole component, 2) Increasing SDHI dose will reduce selection for azole resistant mutants and 3) there will be an additional benefit from reducing dose of azole component.

Table 3. Azole sensitivity profiles of *Zymoseptoria tritici* field strains carrying different CYP51 variants. Data presented as resistance factors in comparison with the azole sensitivities determined for four wildtype CYP51 variant strains.

CYP51 variant[1]	Number of strains	Resistance factor[2]		
		Epoxiconazole	Prochloraz	Tebuconazole
[Y137F]	5	6.0	5.1	3.5
[Y137F, S524T]	5	40	33	12
[L50S, Y461S	3	19	9.4	21
[L50S, Y461H]	2	17	4.3	16
[L50S, I381V, Y461H]	36	74	3.6	36
[L50S, I381V, Y459D]	12	56	2.1	36
L50S, V136A, Y461H]	11	55	25	4.8
[L50S, V136A, Y461S]	2	64	31	1.9
[V136A, S188N, Δ]	4	40	25	1.2
[L50S, V136A, Y461S, S524T]	4	215	55	4.0
[L50S, S188N, Δ, N513K]	4	31	4.2	31
[L50S, V136A, S188N, Δ, N513K]	3	89	22	1.6
[L50S, V136A, S188N, Δ, S524T]	6	168	62	5.7
[L50S, D134G, V136A, I381V, Y461H]	35	196	11	5.0
[L50S, S188N, I381V, Δ, N513K]	4	86	3.2	28
[L50S, S188N, I381V, Δ, N513K]↑	19	389	17	153
[L50S, V136A, S188N, Δ, N513K]	6	168	62	5.7
[L50S, S188N, A379G, I381V, Δ, N513K]	48	149	1.3	82
[L50S, D134G, V136A, I381V, Y461H, S524T]	6	809	18	11

[1] Δ indicates absence of amino acid residues Y459 and G460 through a 6 bp deletion in *CYP51*
[2] Resistance factors of strains belonging to a specific CYP51 variant were calculated as the fold change in mean EC_{50} compared to the mean EC_{50} of four wildtype CYP51 strains carrying no amino acid substitutions. Mean EC_{50} values of the wild-type CYP51 variants were 0.003, 0.016 and 0.072 mg L^{-1} for epoxiconazole, prochloraz and tebuconazole, respectively
↑, CYP51 over-expressing strains based on presence of 120 bp *CYP51* promoter insert reported by Cools *et al.* (2012)

Table 4. Fungicide treatment list with spray timings for the 2010/11 growing season

Treatment	Autumn	Pre-T1 GS30-31	T1 (Leaf 3 emerged)	T2 (Flag emerged)
1	Untreated	Untreated	Opus 0.5 L ha^{-1}	Opus 0.5 L ha^{-1}
2	Untreated	Untreated	Opus 0.5 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}	Opus 0.5 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}
3	Untreated	Untreated	Opus 0.5 L ha^{-1} + Isopyrazam 0.5 L ha^{-1}	Opus 0.5 L ha^{-1} + Isopyrazam 0.5 L ha^{-1}
4	Untreated	Untreated	Opus 0.5 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}	Opus 0.5 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}
5	Untreated	Untreated	Opus 0.5 L ha^{-1} + Isopyrazam 2.0 L ha^{-1}	Opus 0.5 L ha^{-1} + Isopyrazam 2.0 L ha^{-1}
6	Untreated	Folicur 1.0 L ha^{-1}	Poraz 0.45 L ha^{-1}	Poraz 0.45 L ha^{-1}
7	Untreated	Folicur 1.0 L ha^{-1}	Poraz 0.45 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}	Poraz 0.45 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}
8	Untreated	Folicur 1.0 L ha^{-1}	Poraz 0.45 L ha^{-1} + Isopyrazam 0.50 L ha^{-1}	Poraz 0.45 L ha^{-1} + Isopyrazam 0.50 L ha^{-1}
9	Untreated	Folicur 1.0 L ha^{-1}	Poraz 0.45 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}	Poraz 0.45 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}
10	Untreated	Folicur 1.0 L ha^{-1}	Poraz 0.45 L ha^{-1} + Isopyrazam 2.0 L ha^{-1}	Poraz 0.45 L ha^{-1} + Isopyrazam 2.0 L ha^{-1}
11	Untreated	Untreated	Opus 1.0 L ha^{-1}	Isopyrazam 1.0 L ha^{-1}
12	Untreated	Folicur 1.0 L ha^{-1}	Poraz 0.9 L ha^{-1}	Isopyrazam 1.0 L ha^{-1}
13	Untreated	Untreated	Untreated	Untreated
14	Untreated	Untreated	Untreated	Untreated
15	Untreated	Untreated	Poraz 0.45 L ha^{-1}	Untreated
16	Untreated	Folicur 1.0 L ha^{-1}	Untreated	Untreated
17	Untreated	Folicur 1.0 L ha^{-1}	Untreated	Untreated
18	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}	Untreated	Untreated
19	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}	Untreated	Untreated
20	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}	Poraz 0.225 L ha^{-1}	Untreated
21	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}	Poraz 0.45 L ha^{-1}	Untreated
22	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}	Poraz 0.9 L ha^{-1}	Untreated
23	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}	Poraz 1.8 L ha^{-1}	Untreated
24	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}	Isopyrazam 0.25 L ha^{-1}	Untreated
25	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}	Isopyrazam 0.5 L ha^{-1}	Untreated
26	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}	Isopyrazam 1.0 L ha^{-1}	Untreated
27	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}	Isopyrazam 2.0 L ha^{-1}	Untreated

Table 5. Fungicide treatment list with spray timings for the 2011/12 growing season

Treatment	Pre-treatment (4 weeks pre-T1)	T1 (Leaf 3 emerged)	T2 (Flag emerged)
1	Folicur 1.0 L ha^{-1}	Poraz 0.9 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}	Poraz 0.9 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}
2	Folicur 1.0 L ha^{-1}	Poraz 0.9 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}	Poraz 0.9 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}
3	Folicur 1.0 L ha^{-1}	Poraz 0.9 L ha^{-1}	Poraz 0.9 L ha^{-1}
4	Folicur 1.0 L ha^{-1}	Poraz 0.45 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}	Poraz 0.45 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}
5	Folicur 1.0 L ha^{-1}	Poraz 0.225 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}	Poraz 0.225 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}
6	Untreated	Folicur 1.0 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}
7	Untreated	Folicur 1.0 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}	Folicur 1.0 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}
8	Untreated	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}
9	Untreated	Folicur 0.5 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}	Folicur 0.5 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}
10	Untreated	Folicur 0.25 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}	Folicur 0.25 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}
11	Untreated	Untreated	Untreated
12	Untreated	Untreated	Untreated
13	Folicur 1.0 L ha^{-1}	Untreated	Untreated
14	Folicur 1.0 L ha^{-1}	Untreated	Untreated
15	Folicur 1.0 L ha^{-1}	Poraz 0.225 L ha^{-1}	Poraz 0.225 L ha^{-1}
16	Folicur 1.0 L ha^{-1}	Poraz 0.45 L ha^{-1}	Poraz 0.45 L ha^{-1}
17	Folicur 1.0 L ha^{-1}	Poraz 1.8 L ha^{-1}	Poraz 1.8 L ha^{-1}

Table 6. Fungicide treatment list with spray timings for the 2012/13 growing season

Treatment	T1 (Leaf 3 fully emerged)	T2 (Leaf 1 fully emerged)
1	Opus 1.0 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}	Opus 1.0 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}
2	Opus 1.0 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}	Opus 1.0 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}
3	Opus 1.0 L ha^{-1}	Opus 1.0 L ha^{-1}
4	Opus 0.5 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}	Opus 0.5 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}
5	Opus 0.25 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}	Opus 0.25 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}
6	Folicur 1.0 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}
7	Folicur 1.0 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}	Folicur 1.0 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}
8	Folicur 1.0 L ha^{-1}	Folicur 1.0 L ha^{-1}
9	Folicur 0.5 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}	Folicur 0.5 L ha^{-1} + Isopyrazam 0.25 L ha^{-1}
10	Folicur 0.25 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}	Folicur 0.25 L ha^{-1} + Isopyrazam 1.0 L ha^{-1}
11	Untreated	Untreated
12	Untreated	Untreated

2.3.2 Locations core field trials

The core field trials carried out by SAC were were done at location Balruddery, near Dundee (season 2010/11) and at Stanley (Perthshire) (2011/12 and 2012/13) using cv. Consort. The ADAS field trials were carried out at Rosemaund (Herefordshire) during all 3 growing seasons using cv. Consort. The trials conducted by Velcourt were carried out near Dover (Kent) at Waldershare Park Farm (2010/11 and 2011/12) and at Martin Lodge Farm (2012/13) using cultivars Cordiale and Solstice, respectively. A randomised block design, incorporating all treatments with three (ADAS and SAC) or four (Velcourt) replicate plots in the range 4 to 10 metres in length and 1.5 to 2 metres width was used for all core field trials.

2.3.3 Rothamsted Farm, commercial fields and Fungicide performance winter wheat trials 2014-18

Leaves with Septoria symptoms were sampled each year at locations where the Fungicide Performance against Septoria were measured (see Bounds *et al*., 2016). The locations with Septoria trials are: Fife (Scotland) run by SRUC, Sutton Scotney (Hants) (NIAB-TAG), Rosemaund (Herefordshire) (ADAS), Cardigan (Wales) (ADAS) and Carlow (Ireland) Teagasc. Samples (50 leaves with Septoria symptoms) were taken at the start of the season before spraying (March-April) and 3-4 weeks after a single T1 or T2 spray (20 leaves with symptoms per replicate plot (3 or 4 replicate plots were sampled)) with a full dose of an azole (epoxiconazole (Opus Max or

Ignite)), a SDHI (fluxapyroxad (Imtrex)) or an azole. An untreated field was also sampled each year at Rothamsted during January-February (50 leaves). In addition, several fields from farms operated by Velcourt, ADAS, SRUC and NIAB-TAG were also sampled at the start of the season and the end of the season each year during 2014-2018.

2.3.4 Sampling and assessments

Factors to be measured in trials were levels of disease control, frequency of CYP51 alterations and *in vitro* sensitivity of a selection of strains at key sampling dates. A full disease and GLA assessment (on 10 plants) was carried out at all sampling timings. Trials were sampled before spraying (100 leaves per field) and 3 weeks after the T1 and T2 spray (25 leaves per plot of highest leaf layer with clear symptoms, most likely Leaf 3 or 4 after the T1 spray and Leaf 1 or 2 after the T2 spray). Sampled leaves were wrapped in dry tissue paper and sent in paper envelopes to Rothamsted Research for *CYP51* sequence analysis, strain isolation and fungicide sensitivity testing. For each whole field sample of 100 leaves or plot sample of 25 leaves, 50 ng of total DNA was tested in the SNP detection Pyrosequencing assays. For a selection of pre- and post-treatment leaf samples, strains of *Z. tritici* were isolated and their sensitivity to different azoles (i.e. epoxiconazole, prochloraz, tebuconazole and prothioconazole-desthio) and the SDHI bixafen determined. DNA from azole insensitive isolates was further analysed by *CYP51* gene sequencing to establish phenotype-to-genotype (CYP51 variant) relationships and to check for presence of other resistance mechanisms.

2.3.5 Industry partner field trials and SDHI resistance surveys

All agrochemical industry partners conducted field trials annually to test the effects of different fungicide programmes on disease control and azole insensitivity development in populations of *Z. tritici*. The design of these experiments included some treatments and the same sampling protocol from the core field trial experiments to aid comparison. The industry partners also provided data on the geographic distribution of azole resistance in European *Z. tritici* populations and additional information on choice and timing of azole-based spray programmes. In addition, they also carried out monitoring of azole performance and SDHI resistance development in other key cereal pathogens (e.g. *Ramularia collo-cygni*, *Pyrenophora teres* and *Rhynchosporium commune*) within the UK and more widely across Europe.

2.4 Results

2.4.1 SDHI fungicide base-line sensitivity testing of *Zymoseporia tritici* field strains up to 2010

Table 7 shows the fungicide sensitivities of 30 field strains representing past and recent field populations sampled from different geographic regions. Carboxin was the least effective compound amongst the SDHIs tested, with recorded EC_{50} values between 0.608 and 5.52 µg mL^{-1} and an average value of 1.84 µg mL^{-1}. Boscalid (EC_{50}s between 0.161 and 1.14 µg mL^{-1}; average of 0.482 µg mL^{-1}) was less effective *in vitro* than isopyrazam (EC_{50}s between 0.042 and 1.11 µg mL^{-1}; average of 0.262 µg mL^{-1}) and bixafen (EC_{50}s between 0.019 and 0.662 µg mL^{-1}; average of 0.219 µg mL^{-1}). When sensitivity data from strains isolated before 2005 were compared with sensitivities of those isolated after 2005 (coinciding with the introduction of boscalid in the UK), there were no differences in EC_{50} values for the four SDHIs and chlorothalonil. The sensitivity data for epoxiconazole and prothioconazole-desthio showed a clear trend of decreasing sensitivity for strains isolated more recently in NW-Europe, a region with a high Septoria disease pressure, compared to older strains. This change in sensitivity was correlated with the evolution of more complex CYP51 variants harbouring multiple amino acid alterations.

Clear linear cross-resistance patterns were observed for bixafen and isopyrazam, bixafen and boscalid, and isopyrazam and boscalid with R^2 values of 0.65 (p<0.001), 0.61 (p<0.001) and 0.46 (p<0.001) (n=30), respectively. The R^2 values for carboxin and bixafen, carboxin and isopyrazam and carboxin and boscalid were lower with values of 0.17 (p=0.022), 0.17 (p=0.025) and 0.03 (p=0.385) (n=30), respectively. As expected, of the remaining fungicide pairs tested, a clear linear cross-resistance relationship was only measured between the two DMI fungicides epoxiconazole and prothioconazole-desthio (R^2=0.85 (p<0.001), n=21).

Table 7. Characterisation of *Zymoseptoria tritici* field strains.

Isolates	CYP51 amino acid alterations[1]	Origin	Year	Fungicide sensitivity (EC$_{50}$ in µg mL^{-1})[2]						
				Car-boxin	Bosca-lid	Bixa-fen	Iso-pyrazam	Epoxi-conazole	Prothio-desthio	Chloro-thalonil
IPO323	none	Netherlands	1981	1.50	0.970	0.273	0.418	<0.0005	0.0018	0.167
IPO88004	none	Ethiopia	1988	1.43	0.208	0.031	0.107	<0.0005	0.0009	0.145
A12/3B/8	none	USA	1988	1.99	0.210	0.055	0.053	<0.0005	0.0015	0.191
IPO90012	none	Mexico	1990s	2.05	0.300	0.086	0.073	<0.0005	0.0013	0.119
IPO001	none	Netherlands	1991	1.27	0.203	0.054	0.078	<0.0005	0.0015	0.141
RD14	Y137F	UK	1993	0.622	0.271	0.094	0.119	0.0057	0.0060	0.040
ST16	L50S, G460D	UK	1995	1.61	0.603	0.381	0.539	0.0018	0.0023	0.145
IPO92006	L50S, S188N, N513K	Portugal	1990s	3.75	0.615	0.393	0.169	<0.0005	0.0015	0.218
CTRL01-1	Y137F, S524T	UK	2001	3.13	0.438	0.130	0.176	0.0536	0.0042	0.097
LARS15	L50S, I381V, Y459D	UK	2003	1.40	0.433	0.175	0.247	0.0549	0.0105	0.109
G303	L50S, S188N, A379G, I381V, Δ, N513K	UK	2003	2.25	1.14	0.664	0.591	0.0749	0.0134	0.176
IRE13	L50S, V136A, Y461S	Ireland	2003	0.868	0.197	0.030	0.042	0.0268	0.0127	0.069
IRE30	L50S, V136A, Y461S	Ireland	2003	1.75	0.522	0.090	0.130	0.0399	0.0110	0.162
POL27	L50S, I381V, Y461H	Poland	2004	1.27	0.524	0.207	0.397	0.127	0.0144	0.155
NZ75	Y137F	New Zealand	2004	1.20	0.292	0.057	0.074	0.0056	0.0011	0.235
BD04	G460D	Spain	2006	0.711	0.655	0.127	0.435	0.0056	0.0023	0.127
MM20	none	Spain	2006	3.42	0.394	0.383	0.368	<0.0005	0.0018	0.210
V212-2	V136A, S188N, Δ	UK	2006	2.69	0.415	0.215	0.184	0.0712	0.0118	0.141
4439	none	Finland	2007	0.765	0.604	0.312	0.410	<0.0005	0.0006	0.165
T2	D107V, I381V, N513K, S524T	France	2008	0.608	0.446	0.179	0.294	0.0578	0.0063	0.113
NZ11-1	L50S, S188N, N513K	New Zealand	2008	3.56	0.454	0.078	0.130	<0.0005	0.0019	0.212
I43-2	L50S, V136A, Y461S, S524T	Ireland	2009	1.10	0.630	0.391	0.206	0.244	0.0514	0.104
R35-3	L50S, I381V, Y461H	UK	2009	1.71	0.375	0.183	0.279	0.0910	0.0193	0.254
R39-1	L50S, S188N, A379G, I381V, Δ, N513K	UK	2009	5.52	0.795	0.662	1.10	0.278	0.0262	0.325
BC1	L50S, I381V, Y461H	UK	2009	1.64	0.269	0.095	0.210	0.0818	0.0155	0.316
BC4	L50S, I381V, Y461H	UK	2009	1.42	0.472	0.408	0.328	0.151	0.0270	0.329
TAG71-3	L50S, D134G, V136A, Y461S, S524T	UK	2009	2.05	0.161	0.019	0.020	0.453	0.156	0.336
TAG1-9	L50S, V136A, I381V, Y461H, S524T	UK	2010	1.35	0.498	0.271	0.206	1.50	0.180	0.081
TAG74-3	L50S, S188N, I381V, Δ, N513K	UK	2010	1.46	0.305	0.115	0.123	1.05	0.167	0.151
ROS1.40.1	L50S, V136A, I381V, Y461S, S524T	UK	2010	1.24	0.522	0.402	0.130	0.658	0.152	0.146

[1] Δ indicates deletion of both Y459 and G460 (6 bp deletion)
[2] Fungicide sensitivity values (EC$_{50}$s) are the mean of two measurements.

2.4.2 *Sdh* sequence variation among *Zymoseptoria tritici* isolates up to 2010

DNA sequence comparison between IPO323, IRE30 and four other strains (BC1, BC4, R35-3, R39-1 and V212-2) showed that the Sdh subunits were generally well conserved (GenBank accession numbers JF916683-JF916700). For SdhB, only changes resulting in the replacement of lysine (aag) by arginine (agg) at position 48 (B-K48R) and cysteine (tgc) by arginine (cgc) at codon 276 (B-C276R) were found in strains BC1 and V212-2, respectively. Strain 35-3 had a proline (ccc) residue (instead of serine (tcc)) at position 51 in SdhC (C-S51P) and isolate R39-1 had two changes in this subunit, a valine (gtc) residue (instead of isoleucine (gtc)) at codon 29 (C-I29V) and a glycine (ggc) residue (instead of arginine (cgc)) at codon 54 (C-R54G). Two SdhC changes were also found in strain V212-2, with two asparagines (aac) residues replaced by threonine (acc) at codons 33 (C-N33T) and 34 (C-N34T). For SdhD, strain R35-3 carried a glycine (ggt) residue (instead of alanine (gct)) at codon 10 (D-A10G), whereas IRE30 harboured an unique proline (ccg) residue (instead of arginine (cgg)) at codon 47 (D-R47P). *SdhC* was most variable with regard to additional nucleotide changes leading to synonymous substitutions and sequence variation within intron regions (data not shown).

2.4.3 Docking of carboxin and other SDHIs in the quinone binding site of the *Zymoseptoria tritici* Sdh complex

Docking studies (Figures 7 and 8) showed carboxin positioned in a similar way as in the crystal structure of the avian Sdh complex (Huang *et al.*, 2006).

Figure 7. Docking of carboxin and fluopyram in the quinone binding site of the *Zymoseptoria tritici* Sdh complex. The Sdh structural model is shown with the carbon atoms in green, with the residues forming the binding pocket with the carbon atoms in cyan. Carboxin and fluopyram are shown with carbon atoms in magenta and yellow, respectively. Hydrogen bonds are indicated in dashed lines.
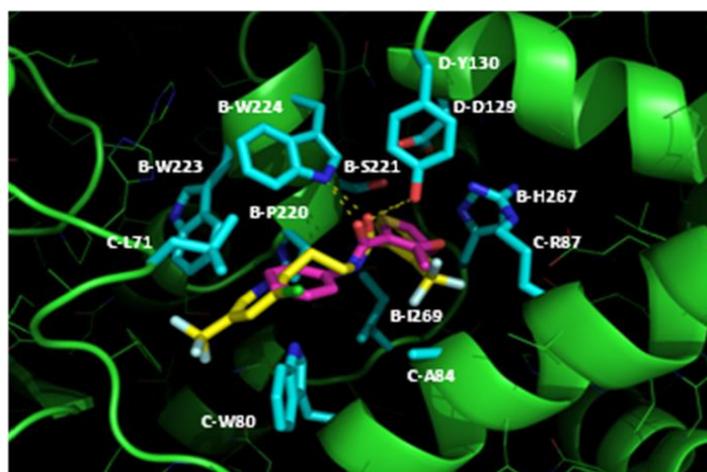
SdhA did not form part of the quinone binding site. SdhB residues P220, S221, W223, W224, H267 and I269 form the major part of the binding pocket. SdhC (L71, W80, S83, A84 and R87) and SdhD (D129 and Y130) form the remainder of the fungicide binding cavity. The docking study also indicates that the binding energy for the carboxin-Sdh complex is relatively high which suggests relatively poor binding when compared with the other fungicides (Table 8). This probably reflects the relatively small size of this molecule, reducing extensive protein-fungicide interactions. Boscalid and bixafen contain an additional phenyl ring compared to carboxin which is reflected in lower binding energies. The predicted binding energies of carboxin, boscalid, bixafen and isopyrazam (Table 8) are perfectly in line with the respective base-line fungicide sensitivity levels (Table 7). The chlorinated phenyl rings of boscalid and bixafen occupy the same binding pocket as the phenyl moiety of docked carboxin (Fig. 8A, 8B and 8C).

Figure 8. Binding of SDHIs to the quinone binding site of the *Zymoseptoria tritici* Sdh complex. Carboxin (A), bixafen (B), boscalid (C), isopyrazam (D), penthiopyrad (E) and fluopyram (F). The electrostatic potential of the protein surface is colored from red (negative charge) to blue (positive charge).
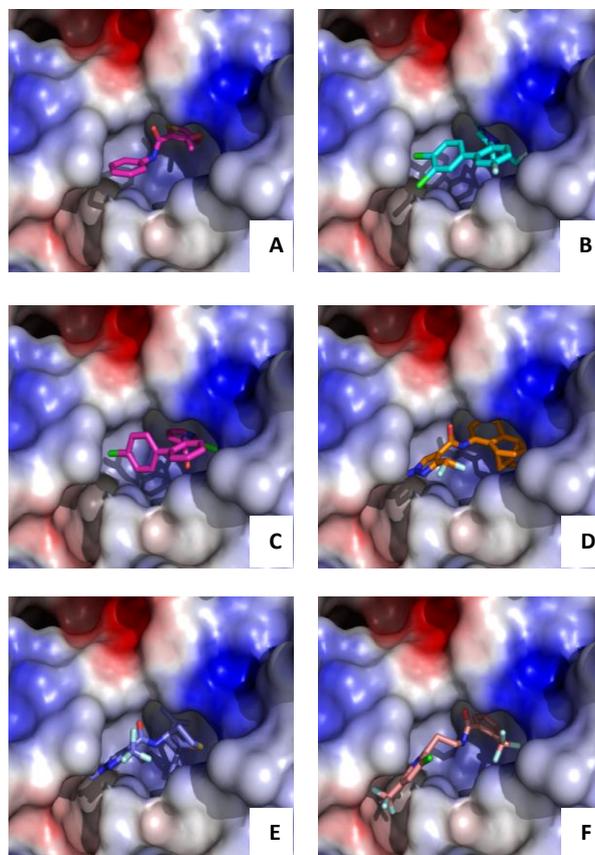
However, the amide moiety is bound in another position, as is the second phenyl moiety of these fungicides that is located at the surface of the protein. The pyrazole and pyridine moieties of bixafen and boscalid are found in the same pocket as the non-aromatic ring of carboxin. Binding seems stronger for bixafen suggesting that the pyrazole moiety interacts favourably when compared with the pyridine moiety of boscalid as both fungicides adopt similar binding modes when docked into the Sdh complex. Docking of isopyrazam (Fig. 8D) and penthiopyrad (Fig. 8E) yielded similar binding energies when compared with bixafen (Table 8). However, the binding mode more closely resembles the binding of carboxin. The pyrazole moieties occupy the same pocket as the phenyl group of carboxin, while their amide and hydrophobic moieties bind in the same location as the amide and the non-aromatic ring of carboxin. To compare the effectiveness of fluopyram *in silico* we also docked this novel fungicide. The predicted fluopyram binding is largely similar to binding of carboxin as the phenyl ring and the amide moiety are in the same location as the non-aromatic ring and amide of carboxin (Fig. 8F).

Table 8. Lowest binding energies obtained by docking of fungicides in a structural model of the wild-type Sdh complex (WT) from *Zymoseptoria tritici* and fungicide-resistance related Sdh variants.

Inhibitor[1]	Binding energies for each complex (kJ mol^{-1})[2]										
	WT	S221P SdhB	R265P SdhB	H267L SdhB	H267Y SdhB	H267N SdhB	I269V SdhB	N86K SdhC	I127V SdhD	D129E SdhD	D129G SdhD
Boscalid	-113.0	-114.9	-114.2	**-111.8**	-112.7	-112.5	-112.8	-114.8	-114.6	-116.1	**-110.5**
Carboxin	-92.0	-92.5	**-90.2**	**-90.0**	-93.1	**-89.1**	**-88.7**	**-90.3**	**-90.5**	-91.1	**-84.0**
Bixafen	-121.9	-129.4	-122.6	-121.8	-122.2	-125.1	**-120.0**	-124.8	-122.7	-121.7	-122.1
Isopyrazam	-122.8	**-115.5**	**-119.8**	**-115.2**	**-113.0**	-126.4	-123.4	**-118.9**	-123.4	**-118.6**	-136.2
Penthiopyrad	-122.4	**-120.5**	-122.2	**-120.2**	**-119.2**	-122.6	**-121.1**	-124.1	-122.2	**-121.1**	-125.6

[1] The *S*-enantiomer of penthiopyrad and the (C6 *S*, C10 *R*, C21 *S*) enantiomer of isopyrazam were used for docking studies.
[2] Higher binding energy values in comparison with the wild-type (WT) Sdh variant indicate a weaker binding of the inhibitor to the target. Values with a significant increase (>1 kJ mol^{-1}) are presented in bold.

However, the other moiety of fluopyram, a benzamide group, is separated from the amide bond by two carbons. This facilitates extended binding of this fungicide as the benzamide moiety is favourably stacked in between two tryptophans, SdhB W223 and SdhC W80 (Fig. 7). There is no such spacer between the amide and the analogous phenyl group in carboxin. This suggests a very tight binding as the binding energy was the lowest of all docked SDHIs (-138.6 kJ mol^{-1}) (Table 8).

2.4.4 Isolation and identification of *Sdh* mutations in carboxin-resistant UV mutants of *Zymoseptoria tritici*

In total 124 mutants, 98 derived from IPO323 (Table 9) and 26 from IRE30 (Table 10), were isolated and characterised. Mutants in the collection had a wide range of non-synonymous mutations (Fig. 9) and corresponding SDHI sensitivity profiles (Tables 9 & 10). Some rare mutants had multiple mutations in the same or in different *Sdh* subunit genes. Figure 6 shows the position of nine key amino acids that were substituted and how conserved these residues are in SdhB, C and D sequences from chicken (*G. gallus*), *A. alternata*, *A. oryzae* and *B. cinerea*. SdhB-C137R is unique for *Z. tritici* and no mutations have been found at or in close proximity to this position in SDHI resistant strains of other fungi. This residue is normally part of a FeS cluster, relatively far from the quinone binding site, and it is expected that such a mutation would result in a non-functional protein. SdhB codon S221 (S221P/T) is one position away from codon 225 in *B. cinerea* where substitutions P225L/F/T have been reported for field strains (Stammler *et al.*, 2008).

Figure 9. Positions of key amino acid alterations in different Sdh subunits from SDHI resistant mutants of *Zymoseptoria tritici*. Partial Sdh subunit sequences are presented for *Z. tritici* (Zt), *A. alternata* (Aa), *A. oryzae* (Ao), *B. cinerea* (Bc), *C. cassiicola* (Cc) and *Gallus gallus* (Gg). Positions of key amino acid residues that have been found altered in SDHI resistant Zt lab mutants are marked with a star. Shaded residues are key amino acid residues that have been altered and linked with SDHI resistance in other pathogens. Bold amino acids are conserved residues.

```
SdhB               *          SdhB          **    *       SdhB           *  *  *
Zt 131-GVNTLACLCRIPT-143      Zt 214-CCSTSCPSYWWNS-226     Zt 261-MSLYRCHTILNCS-273
Aa 141-GVNTLACLCRIPT-153      Aa 224-CCSTSCPSYWWNQ-236     Aa 271-MSLYRCHTILNCT-283
Ao 113-GVNTLACLCRIPT-125      Ao 196-CCSTSCPSYWWNS-208     Ao 243-MSLYRCHTILNCT-255
Bc 136-GVNTLACLCRIPR-148      Bc 219-CCSTSCPSYWWNQ-231     Bc 266-MSLYRCHTILNCT-278
Cc 140-GVNTLACLCRIPT-152      Cc 225-CCSTSCPSYWWNQ-237     Cc 272-MSMYRCHTILNCS-284
Gg 117-GGNTLACTKKIDP-129      Gg 201-CCSTSCPSYWWNG-213     Gg 248-FSLYRCHTIMNCT-260

SdhC        ***   *
Zt 081-YLSALNRVTGVAA-093
Aa 070-YASSLNRITGITL-082
Ao 082-IGSSFHRITGFAL-094
Bc 081-IMSGLNRITGCIL-093
Cc 070-YASSFNRITGVAL-082
Gg 037-AMSITHRGTGVAL-049

SdhD    *    *    * *
Zt 118-HSYIGFQSAITDY-130
Aa 133-HSHIGFESCIIDY-145
Ao 113-HSHIGFQAAIIDY-125
Bc 132-HSHIGFESCITDY-144
Cc 105-HSHIGFEACVIDY-117
Gg 100-HGHWGLGQVITDY-112
```

SdhB codons 265 (R265P), 267 (H267F/L/N/Y) and 269 (I269V) are clustered together and mutations resulting in H267Y/L/N have also been reported in a range of other fungi (Avenot and Michailides, 2010). SdhC N80K reported for *Coprinus cinereus* (Ito *et al*., 2004) is identical to N86K in *Z. tritici*. In addition, SdhC L85P and N86S were also found in *Z. tritici* mutants. SdhC S73P and T90I reported for *C. cassiicola* (Miyamoto *et al*., 2010) and *A. oryzae* (Shima *et al*., 2009), respectively, are within three positions of codons 85 and 86 in *Z. tritici*. SdhD codons 127 (I127V) and 129 (D129E/G/T) are clustered together. SdhD D89G and D124E reported in *Paracoccus nitrificans* (Matsson *et al.*, 1998) and *A. oryzae* (Shima *et al*., 2009), respectively, are equivalent to D129G and D129E in *Z. tritici*. G109V in *C. cassiicola* (Miyamoto et al., 2010) and H133R in *A. alternata* (Avenot *et al*., 2009) are part of the α-helix that positions the two crucial quinone binding site residues in *Z. tritici*, SdhD D129 and Y130. However, they are too far from the quinone binding site (approximately 12 Å) to have a direct effect on fungicide binding.

Table 9. Overview of key mutations and associated amino acid substitutions in Sdh complexes of *Zymoseptoria tritici* strain IPO323 derived mutants (n = 98) and their SDHI sensitivity profiles.

Strains/mutant Sdh variants	Corresponding codon changes	Mutant number	Fungicide sensitivity range (µg mL^{-1})			
			Carboxin[2]	Boscalid	Bixafen	Isopyrazam
IPO323			7.94	0.718	0.216	0.316
B-C137R	tgt > cgt	1	159	>12.5	2.01	1.93
B-S221P[1]	tcc > ccc	1	11.8	0.344	0.014	<0.001
B-S221P[1]	tcc > ccc	5	102 - 190	>12.5	0.761 - 1.91	0.889 - 2.34
B-S221T	tcc > act	2	81.7 - 113	0.959 - 2.0	0.465 - 0.583	0.742 - 1.11
B-R265P	cga > cca	6	133 - 175	3.26 - 5.0	0.235 - 1.02	0.644 - 1.83
B-H267F	cac > ttc	1	58.1	>12.5	2.2	3.13
B-H267L	cac > ctt/ctc	9	>200	>12.5	>12.5	10.0 - >12.5
B-H267N	cac > aac	5	81.0 - 131	0.662 - 1.33	0.284 - 0.787	0.451 - 2.09
B-H267Y	cac > tac	48	67.7 - 193	>12.5	0.482 - 3.05	0.232 - 4.6
B-I269V	att > gtt	7	63.5 - 143	1.1 - 2.86	0.397 - 1.03	1.15 - 1.86
B-S221P, C-R54G	tcc > ccc, cgc > ggc	1	102	2.65	0.462	1.08
B-H267Y, C-N86S	cac > tac, aac > agc	1	153	>12.5	1.46	2.11
B-D166G, D-D129G	gac > ggc, gac > gga	1	>200	>12.5	4.62	5.13
B-I13V, D-D129E, D-V154A	att > gtt, gac > gaa, gtt > gct	1	40.0	3.08	0.969	1.44
C-L85P	ctc > ccc	1	85.1	1.64	0.792	0.669
C-N86K	aac > aaa	1	112	>12.5	>12.5	>12.5
D-I127V	atc > gtc	1	85.1	1.86	0.433	1.27
D-D129E	gac > gag/gaa	5	85.1 - 148	11.6 - >12.5	0.412 - 2.15	0.666 - 4.07
D-D129T	gac > acc	1	>200	3.41	3.49	3.6

[1]Mutants with B-S221P showed two different phenotypes. One mutant derived from IPO323 (M152) was extreme sensitive to all SDHIs tested. The other B-S221P mutants (n =5) were resistant to all SDHIs tested.
[2]The carboxin sensitivity reported for strain IPO323 was higher than that reported in Table 8 due to a wider range of fungicide concentrations tested needed to measure high resistance levels in the mutants.

Table 10. Overview of key mutations and associated amino acid substitutions in Sdh complexes of *Zymoseptoria tritici* strain IRE30 derived mutants (n = 26) and their SDHI sensitivity profiles.

Strains/mutant Sdh variants[1]	Corresponding codon changes	Mutant number	Fungicide sensitivity range (µg mL^{-1})[2]			
			Carboxin	Boscalid	Bixafen	Isopyrazam
IRE30			2.82	0.389	0.070	0.088
B-S221P	tcc > ccc	5	57.8-108	0.447-1.09	0.058-0.243	0.096-0.338
B-H267N	cac > aac	4	64.7-99.3	1.11-1.88	0.284 – 0.81	0.398-2.09
B-H267Y	cac > tac	14	69.0-155	>12.5	0.777-2.6	0.882-4.04
B-P155L, B-H267Y	cca > cta, cac > tac	1	23.6	0.878	0.682	0.486
C-L85P, D-V96A	ctc > ccc, gtc > gcc	1	53.1	3.33	0.874	1.75
D-D129E	gac > gag	1	149	>12.5	1.25	2.4

[1] Strain IRE30 and its mutants all carry D-R47P in comparison with strain IPO323 and its mutants.
[2] The carboxin sensitivity reported for strain IRE30 was higher than that reported in Table 8 due to a wider range of fungicide concentrations tested needed to measure high resistance levels in the mutants.

2.4.5 Relationship between mutant Sdh variants and SDHI sensitivity

Table 9 shows the relationship between the different Sdh variants and sensitivity ranges measured for the different SDHI fungicides. Mutants carrying B-S221P clustered into three different phenotypic groups depending on the parental strain. In comparison with IRE30 mutants (Table 10), mutants derived from IPO323 have higher levels of resistance to all SDHIs tested; in particular for boscalid with EC_{50} levels >12.5 µg mL^{-1}. For one IPO323-derived mutant (M152), carrying S221P with no other mutations detected in the *SdhB*, *C* and *D* genes, the sensitivity to bixafen, boscalid and isopyrazam was much lower than IPO323. For all other Sdh variants tested there was no parent-dependent difference in SDHI sensitivity. Mutants with multiple mutations in *Sdh* genes (B-P155L/B-H267Y and B-I13V/D-D129E/D-V154A) are more sensitive to carboxin in comparison to mutants carrying Sdh variants with the key single amino acid substitutions (B-H267Y and D-D129E). Mutants carrying variant B-H267L were highly resistant to all four SDHIs tested. High levels of boscalid resistance were measured for variants carrying B-C137R, B-S221P (only IPO323 derived mutants), B-H267F, H267Y, C-N86S, C-N86K, D-D129E and D-D129G. Sensitivity levels to bixafen and isopyrazam were similar for mutants carrying different Sdh variants, with exception of B-I269V mutants that were better controlled with bixafen than isopyrazam. Interestingly, high levels of resistance to both bixafen and isopyrazam were only measured for mutants carrying B-H267L or C-N86K.

2.4.6 *In planta* fitness studies on SDHI-resistant UV mutants of *Z. tritici*

A selection of field strains (BC1, BC4 and IRE30) and Sdh mutants derived from isolate IRE30 were further examined for *in-planta* pathogenicity and the ability to produce spores (Table 11). After 21 days the inoculated areas were completely necrotic and covered with pycnidia for the three field isolates. All mutants produced lesions with pycnidia, but some areas of green leaf tissue were still observed within the inoculated areas. The largest areas of necrosis were observed for mutants M74 and M79 (data not shown). In comparison with IRE30 where 36 ng of pathogen DNA was detected per 50 ng of total DNA after 25 days, the levels of pathogen DNA were lower for the mutants with values between 5 and 30 ng measured. However, half of the mutants tested produced more pycnidiospores than IRE30 after 21 days. The amounts of spores harvested per leaf after 21 days was at least 5-fold higher than the initial amounts of spores inoculated for all mutants and strains tested. Although some small differences in symptom expression, fungal growth and spore production were

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measured for individual mutants, these differences were not linked to a particular Sdh variant (e.g. B-S221P, B-H267N, B-H267Y and D-D129E).

Table 11. *In planta* fitness studies on field strains and SDHI-resistant laboratory mutants of *Zymoseptoria tritici*.

Strains/ mutants[1]	SDHI resistant Sdh alterations[2]	Amount of DNA (pg)[3]				Amount of spores[4]
		7 d	14 d	21 d	25 d	
BC1	no	43 ± 43	2544 ± 17	6047 ± 1668	32270 ± 1750	3.67E+05
BC4	no	334 ± 50	1625 ± 774	7419 ± 1663	29010 ± 2000	3.58E+05
IRE30	no	184 ± 44	100 ± 1.8	17135 ± 615	36245 ± 1495	3.00E+05
M77	B-S221P	248 ± 49	1658 ± 487	1968 ± 114	15330 ± 360	3.33E+05
M99	B-S221P	57 ± 12	148 ± 15	10749 ± 791	17115 ± 145	2.67E+05
M125	B-S221P	169 ± 0.5	100 ± 29	4556 ± 941	5334 ± 2168	2.17E+05
M73	B-H267N	281 ± 7.6	73.4 ± 2.3	12140 ± 860	21045 ± 2015	5.67E+05
M126	B-H267N	143 ± 16	229 ± 48	3174 ± 205	14630 ± 440	6.50E+05
M74	B-H267Y	290 ± 45	1482 ± 74	12500 ± 890	16115 ± 275	4.17E+05
M79	B-H267Y	252 ± 31	419 ± 104	8086 ± 81	29770 ± 3310	2.92E+05
M80	B-H267Y	63 ± 6.7	189 ± 31	9490 ± 2820	12384 ± 2787	1.42E+05
M121	B-H267Y	256 ± 39	572 ± 3.7	4641 ± 15	9479 ± 1251	4.42E+05
M81	D-D129E	159 ± 22	344 ± 52	6831 ± 812	24370 ± 0	1.33E+05

[1] Mutants (M numbers) are all derived from strain IRE30.
[2] Sdh alterations B-K48R in strain BC1 and D-R47P in IRE30 are not associated with SDHI resistance.
[3] Amount of DNA presented is per 50 ng of total DNA extracted from inoculated 2cm segments of four leaves. Mean values of two measurements ± standard errors are given.
[4] Amount of spores is the mean amount of spores collected per leaf sampled after 21 days.

2.4.7 Core field trial results from the 2010/11 growing season

Due to absence of SDHI resistance, the trials were carried out to test the role of SDHIs in protecting azoles with regard to resistance development. Part of the treatments with appropriate controls applied in the 2010/11 growing season (see Table 4) were designed to reduce the initial frequency of V136A by autumn and pre-T1 treatments using Folicur (tebuconazole), then test for the effects of azole/SDHI mixtures using different ratios of doses and alternation on selection for V136A by prochloraz. Epoxiconazole treatments were also included to test for additional effects of azole/SDHI mixtures and alternation on selection for CYP51 mutations and to reflect a more common practise commercial disease control. The treatments varying for number of pre-sprays (treatments 13 & 14, 16 & 17, 18 & 19) acted as controls to account for variation within a treatment block. Each 'pair' of controls (e.g. treatments 13 & 14) had their randomisation fixed so that they were spread out within and between blocks. Additional treatments were included to obtain dose response data for prochloraz and isopyrazam.

2.4.7.1 Septoria trial at ADAS Rosemaund 2011

The trial at Rosemaund was carried out using 7 by 2 m plots. The crop (cv. Consort) was drilled on 25 September 2010 and received 1st pre-spray, 2nd pre-spray, T1 (GS 32/3) and T2 (GS 39-49) applied on 24 February, 14 March, 3 May and 31 May 2011, respectively. During the trial, disease levels were recorded at T1, T2 and GS 75. Figure 10 shows the mean levels of STB recorded for Leaf 1, L2 and L3 at GS75. Isopyrazam applications were more effective than Poraz (prochloraz) where higher doses only improved disease control marginally.

Figure 10. Mean level of Septoria tritici blotch (STB) severity (%) on different leaves sampled at Rosemaund at GS75, three weeks after the T2 spray, in 2011. See Table 4 for treatments. C0, C1 and C2 are control treatments with no, one or two pre-sprays of Folicur (tebuconazole)
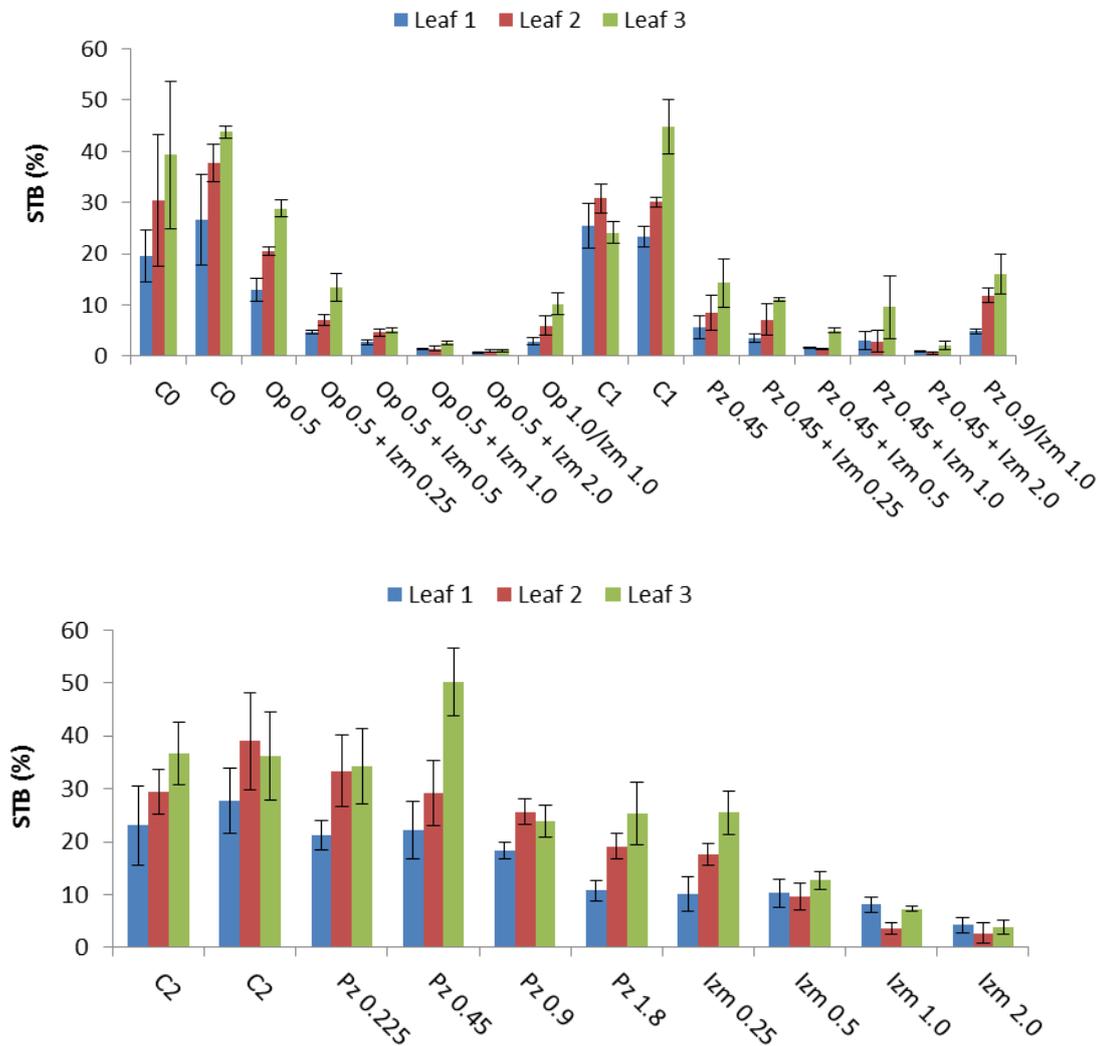
A half dose of Opus (epoxiconazole) was less effective than a half rate of prochloraz. Improved levels of disease control were obtained with isopyrazam as mixing partner. Using identical fungicide inputs, the azole/SDHI mixtures performed much better than the azole to SDHI alternations.

The SNP detection Pyrosequencing assays (Fig. 11) showed that the pre-sprays with tebuconazole (C1 versus C0) selected against V136A and D134G, with frequencies of approximately 40 and 20 %, dropping to low levels (2-5 %) and undetectable levels, respectively.

Figure 11. Selection for CYP51 D134G, V136A, A379A and I381V in untreated and fungicide-treated field populations of *Zymoseptoria tritici* sampled at Rosemaund in 2011. Allele frequencies were measured using SNP detection Pyrosequencing assays. Field populations (Leaf 2 with symptoms) were sampled three weeks after the final spray at GS 75.
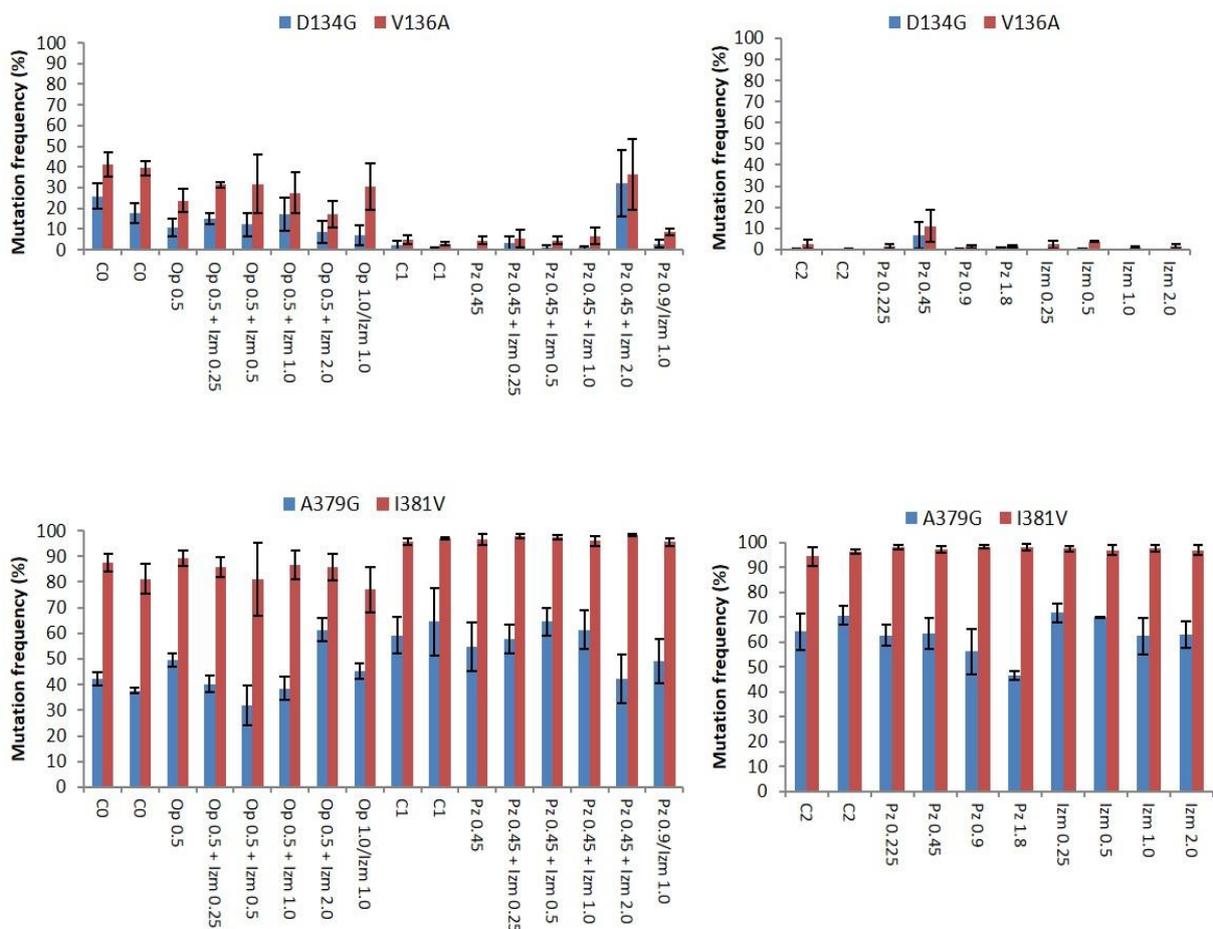
At the same time, increased frequencies of A379G, from 40 to 60 %, and I381V, from 80 to 95 %, were measured. After the epoxiconazole solo treatment (half dose Opus), the frequency of both A379G and I381V alleles increased slightly, whereas the frequencies of D134G and V136A alleles decreased. Adding a quarter, half and full dose of isopyrazam reduced this affect, but not the double dose of isopyrazam in mixture or the alternation treatment of full dose epoxiconazole followed by full dose isopyrazam.

No clear selection for any of the CYP51 mutations was observed after half dose applications of prochloraz solo or in mixture or after solo treatments of isopyrazam (Figure 11). However, higher dose rates of solo prochloraz applications did decrease the frequency of A379G alleles.

At least 40 strains per treatment were isolated from untreated plots (C0), tebuconazole pre-treated untreated plots (C1), plots treated with half dose epoxiconazole, plots treated with half dose of prochoraz, plots treated with mixtures of half dose epoxiconazole and isopyrazam and plots treated with mixtures of half dose prochloraz and isopyrazam. The fungicide sensitivity testing results for all these isolates are displayed in Figure 12. No major shifts in sensitivity were detected with exception of tebuconazole where there was a clear effect of the pre-treatment resulting in a clear selection of less sensitive strains (isolates with $EC_{50} > 1.0$ ppm). A few less extreme sensitive strains for epoxiconazole were identified after both epoxiconazole and prochloraz based treatments. Regarding prochloraz sensitivity, less highly sensitive strains were found after epoxiconazole based treatments and more highly sensitive strains were found after prochloraz based treatments. No effect of isopyrazam was observed when mixtures were compared with solo treatments of prochloraz and epoxiconazole.

Figure 12. Sensitivity of *Zymoseptoria tritici* isolates to epoxiconazole, prochloraz and tebuconazole in populations sampled from untreated and treated plots at Rosemaund in 2011.
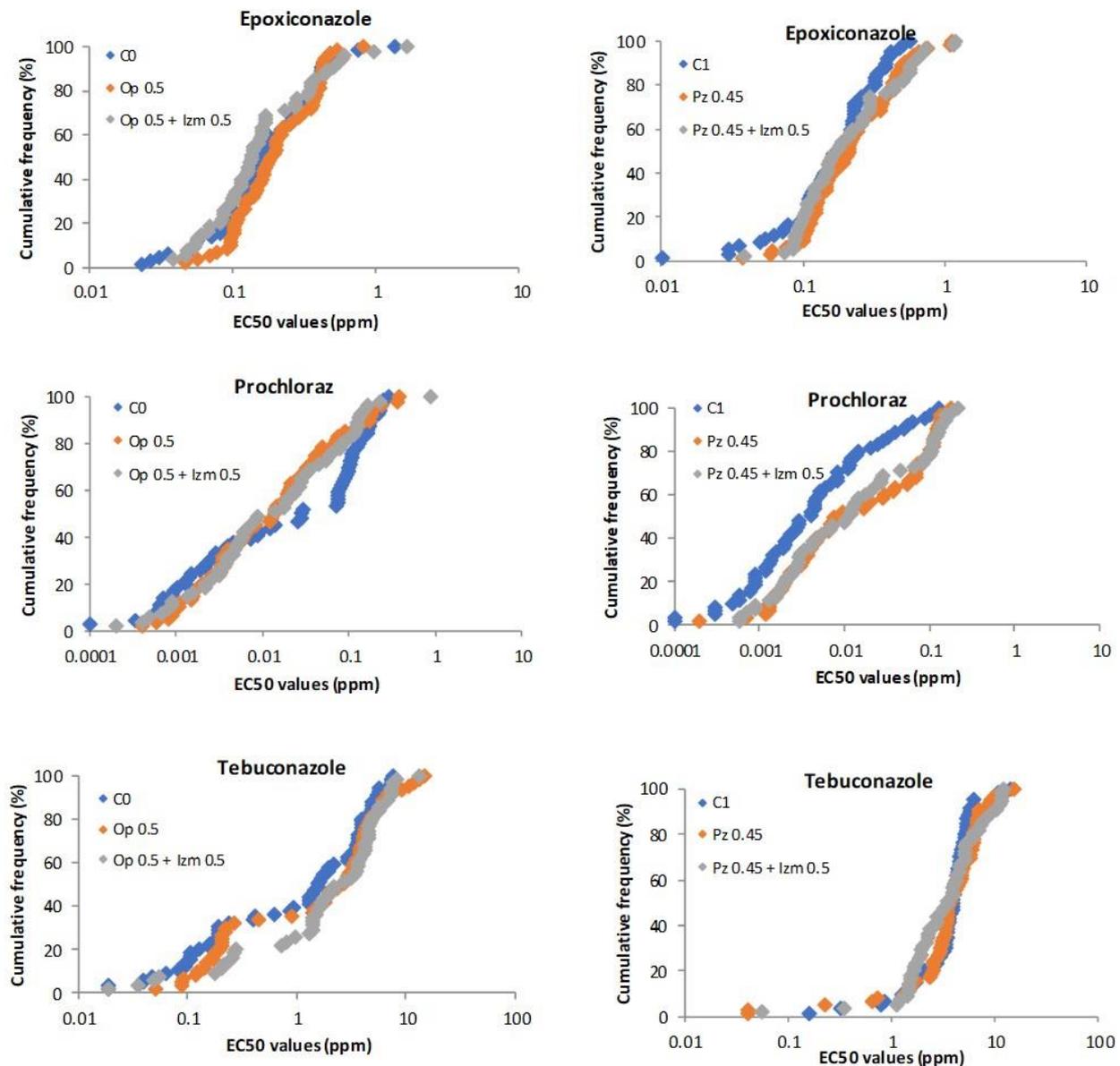
2.4.7.2 SRUC Septoria trial near Dundee in 2011

The trial at the Balruddery site was carried out as planned, using 7 by 2 m plots. The crop (cv. Consort) was drilled on 24 September 2010 and received 1st pre-spray, 2nd pre-spray, T1 (GS 32/3) and T2 (GS 39-49) applied on 24 February, 14 March, 3 May and 31 May 2011, respectively. During the trial, disease levels were recorded at T1, T2 and GS 69. Figure 13 shows the mean levels of STB recorded for Leaf 1, L2 and L3 at GS 69. The efficacy of the isopyrazam applications was erratic for Leaf 1 and Leaf 3, but a clear dose response was obtained for Leaf 2. A clear dose response was also

obtained for with Poraz (prochloraz). A half dose of Opus (epoxiconazole) was less effective than a half rate of prochloraz. Improved levels of disease control on Leaf 2 with Opus were only obtained with isopyrazam as mixing partner at a rate of 2 L Ha^{-1}. For Poraz, an increasing dose of isopyrazam was linked with improved efficacy. Using identical fungicide inputs, the azole/SDHI mixtures performed much better than the azole to SDHI alternations.

Figure 13. Mean level of Septoria tritici blotch (Stb) severity (%) on different leaves sampled near Dundee at GS69, three weeks after the T2 spray, in 2011. See Table 4 for treatments. C0, C1 and C2 are control treatments with no, one or two pre-sprays of Folicur (tebuconazole)
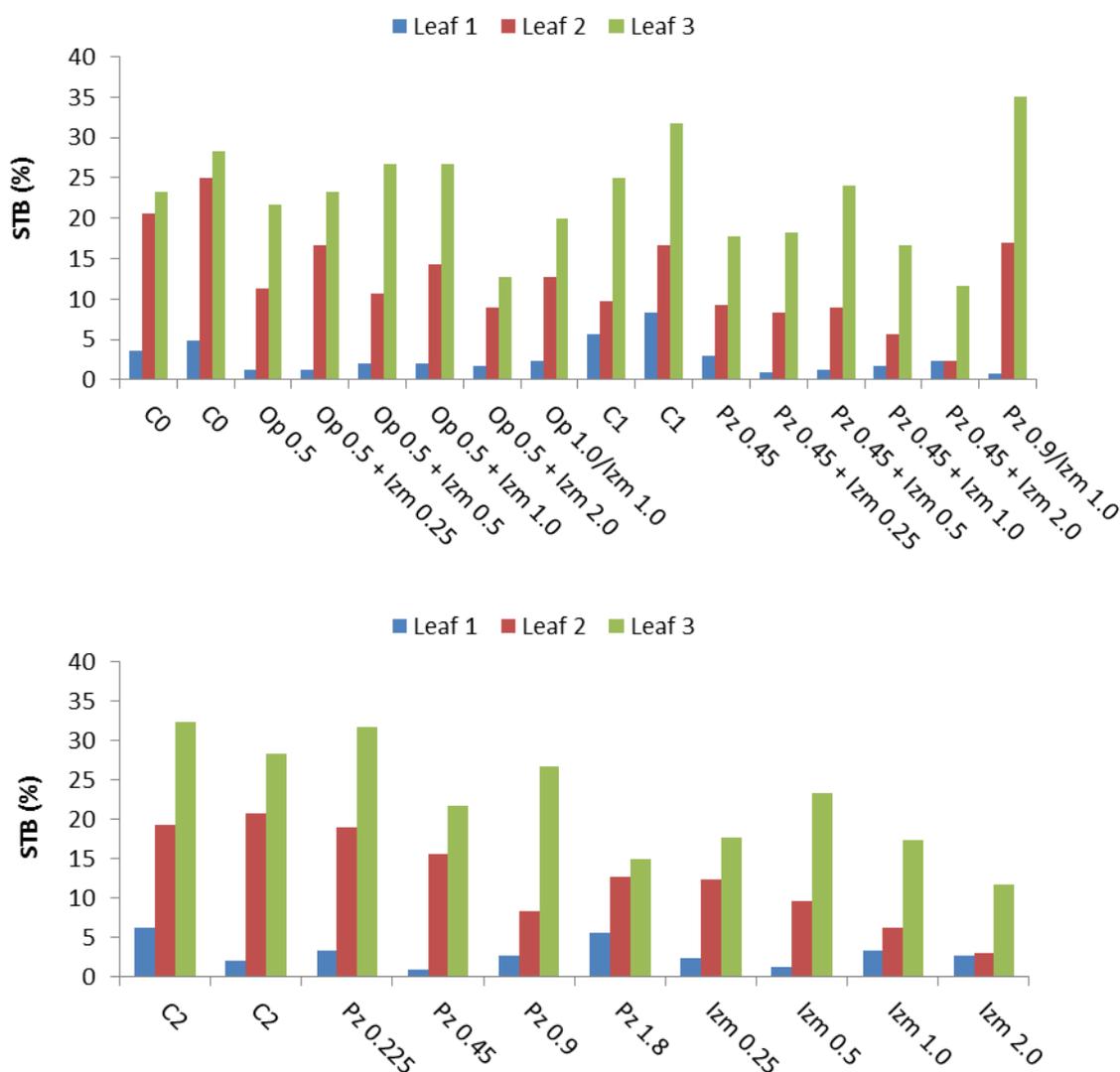
The results of SNP detection Pyrosequencing assays for the leaf samples collected at Dundee are presented in Figure 14.

Figure 14. Selection for CYP51 D134G, V136A, A379A and I381V in untreated and fungicide-treated field populations of *Zymoseptoria tritici* sampled near Dundee in 2011. Allele frequencies were measured using SNP detection Pyrosequencing assays. Field populations (Leaf 2 with symptoms) were sampled three weeks after the final spray.
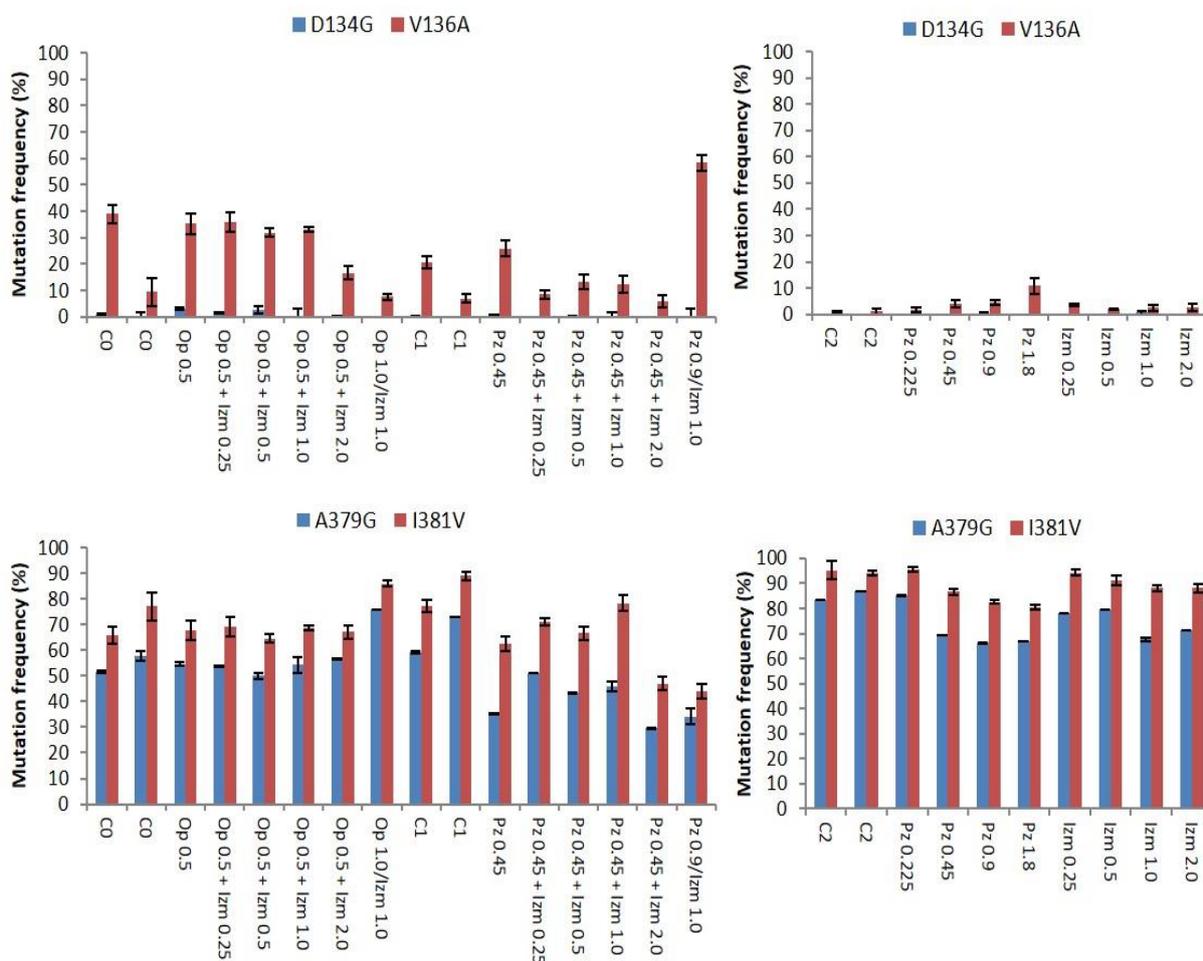
In contrast to Rosemaund, no significant levels of D134G alleles were detected. Increased frequencies of A379G, from 55 to 85 %, and I381V, from 70 to 95 %, were measured after two tebuconazole pre-spray treatments. Increasing doses of prochloraz selected for V136A and against A379A and I381V. Low levels of D134G, up to 5 %, were detected after epoxiconazole treatments but these levels decreased, or alleles were not detected using isopyrazam as mixing partner at the highest rates. The frequency of V136A decreased after the mixture treatment of epoxiconazole at 0.5 L ha^{-1} and isopyrazam at 2.0 L ha^{-1} and the alternation of epoxiconazole (Opus) at 1.0 L ha^{-1} and isopyrazam at 1.0 L ha^{-1}. Higher levels of A379G and I381V were also detected after alternation of epoxiconazole at 1.0 L ha^{-1} and isopyrazam at 1.0 L ha^{-1}. The selection for V136A upon prochloraz treatment slowed down with isopyrazam as mixing partner with only 6 % of V136A alleles measured after mixture applications of

0.45 L ha^{-1} prochloraz and 2.0 L ha^{-1} isopyrazam. The highest mean frequency of V136A alleles (58 %) was measured after alternation of prochloraz at 0.9 L ha^{-1} and isopyrazam at 1.0 L ha^{-1}. Lowest levels of A379A and I381V alleles, down to approximately 30 and 45 %, respectively, were detected after mixture applications of 0.45 L ha^{-1} prochloraz and 2.0 L ha^{-1} isopyrazam and the alternation of prochloraz at 0.9 L ha^{-1} and isopyrazam at 1.0 L ha^{-1}. Solo applications of isopyrazam treatments did not show selection for any of the CYP51 mutations tested.

At least 40 strains per treatment were isolated from untreated plots (C0), tebuconazole pre-treated untreated plots (C1), plots treated with half dose epoxiconazole, plots treated with half dose of prochloraz, plots treated with mixtures of half dose epoxiconazole and isopyrazam and plots treated with mixtures of half dose prochloraz and isopyrazam. The fungicide sensitivity testing results for all these isolates are displayed in Figure 15. In comparison with the isolates sampled at Rosemaund, a few clear differences were observed. The single tebuconazole treatment (pre-spray) did not result in a tebuconazole sensitivity shift. The epoxiconazole based treatments resulted in higher levels of prochloraz insensitive strains (isolates with EC$_{50}$ > 0.01 ppm). Prochloraz based treatments selected for more prochloraz insensitive strains and tebuconazole sensitive strains (isolates with EC$_{50}$ < 1.0 ppm). Mixing isopyrazam with prochloraz reduced the selection for more prochloraz insensitive strains and tebuconazole sensitive strains.

2.4.7.3 Velcourt Septoria trial near Dover in 2011

The trial at Waldershare Park Farm near Dover (Kent) was carried out as planned, using 7 by 2 m plots. The crop (cv. Cordiale) was drilled on 8 October 2010 and received 1st pre-spray 7 weeks before T1 (22 February 2011), 2nd pre-spray 4 weeks before T1 (16 March), T1 (GS 31-32 on 22 April) and T2 (GS 37-39 on 13 May). During the trial, disease levels were recorded at T1, T2 and GS 75. This trial experienced very low levels of diseases as shown in Figure 16.

Figure 15. Sensitivity of *Zymoseptoria tritici* isolates to epoxiconazole, prochloraz, prothioconazole and tebuconazole in populations sampled from untreated and treated plots near Dundee in 2011.
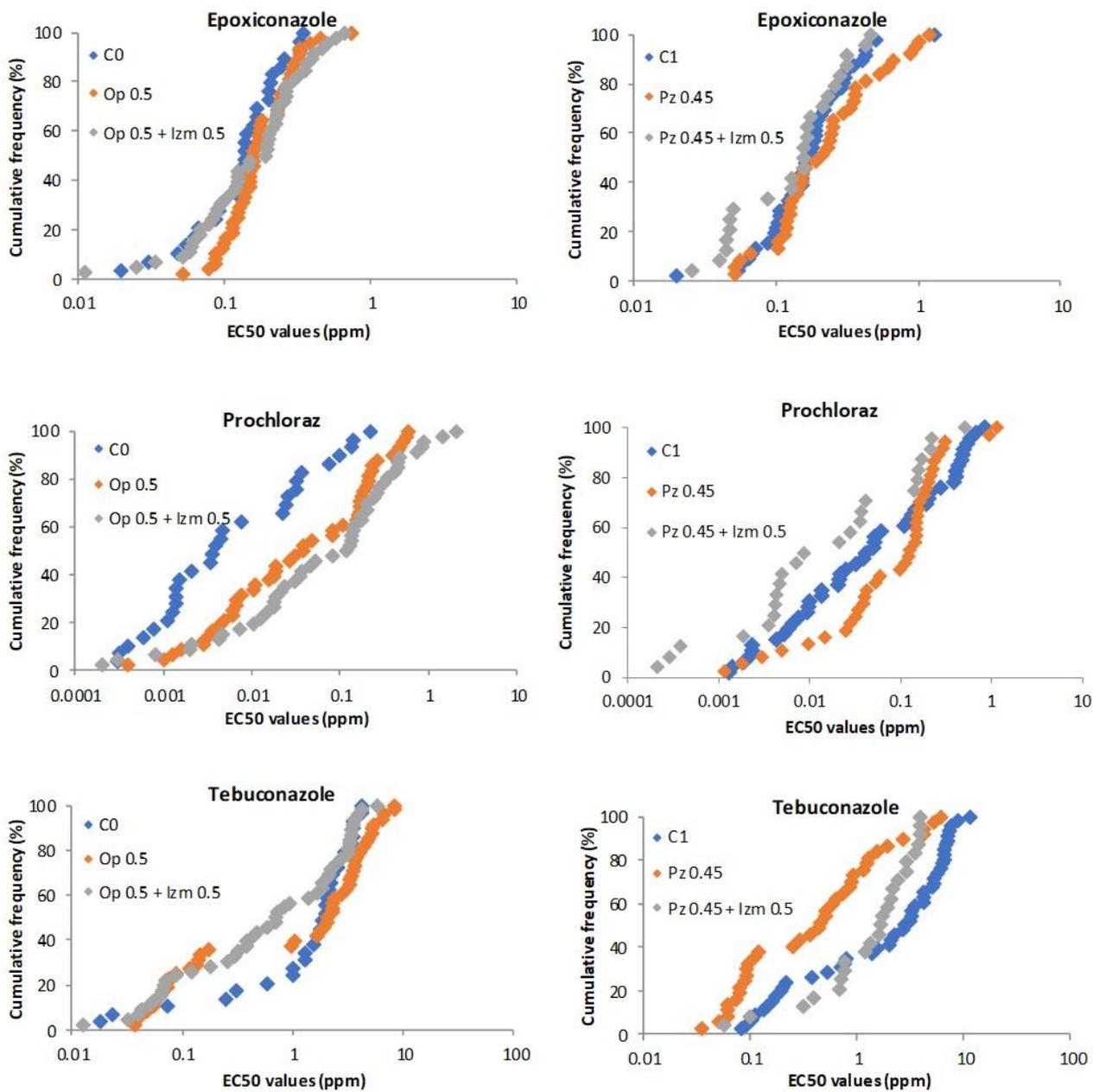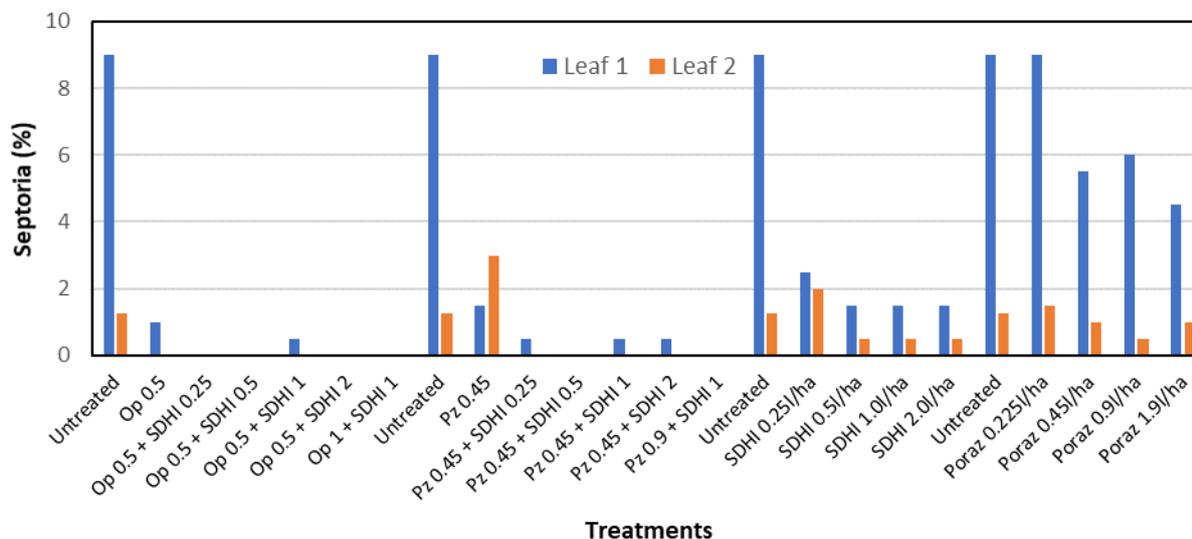
Figure 16. Mean level of Septoria tritici blotch (Stb) severity (%) on different leaves sampled near Dover at GS 75, 4 weeks after the T2 spray, in 2011. See Table 4 for treatments.

Half rate of Opus (epoxiconazole) provided good Septoria control as well as all isopyrazam based treatments, applied solo or in mixture with azoles. Poraz (prochloraz) was less effective, with no control at a quarter dose and only up to 50 % disease control on the flag using a double dose. Due to the low levels of disease, many samples did not produce a PCR product and SNP detection pyrosequencing assays could not be carried out.

2.4.8 Core field trial design for the 2011/12 growing season

After obtaining the results of the previous season, the following treatment changes were introduced into the core field trial design for the 2011/12 growing season:

• Selection against a mutation is a valid test and because of its strong selection, tebuconazole against V136A was used to test mixtures with SDHI fungicides (SDHIs being used to protect azole against resistance development in the absence of SDHI resistance conferring mutations)

• Size of mixture effect should increase by lower doses of azole with higher doses of SDHI. Add mixture treatments covering a range of doses of azole and SDHI (guided by dose-response curves to maintain constant efficacy)

• To make room for these treatments, isopyrazam dose response treatments were removed (data available from HGCA trials) as well as double pre-treatment controls and epoxiconazole mixtures

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2.4.8.1 Septoria trial at ADAS Rosemaund 2012

The trial at Rosemaund was carried out as planned, using 7 by 2 m plots. The crop (cv. Consort) was drilled on 10 September 2011 and received a pre-spray, 4 weeks prior T1 (GS 30 on 21 March), T1 spray (GS 33 on 5 April) and T2 spray (GS 39 on 25 May). During the trial, disease levels were recorded at T1, T2 and GS 75. Figure 17 shows the mean levels of Stb on Leaf 3 at GS75.

Figure 17. Mean level of Septoria tritici blotch severity (%) on Leaf 3 at location Rosemaund on 25th of May (T2) in 2012 (P<0.001). Pre-spray (4 weeks prior T1) with tebuconazole (Folicur 1.0 L/ha) was carried out for all prochloraz based treatments and untreated C1 plots (see Table 5 for all treatments applied at T1 and T2).
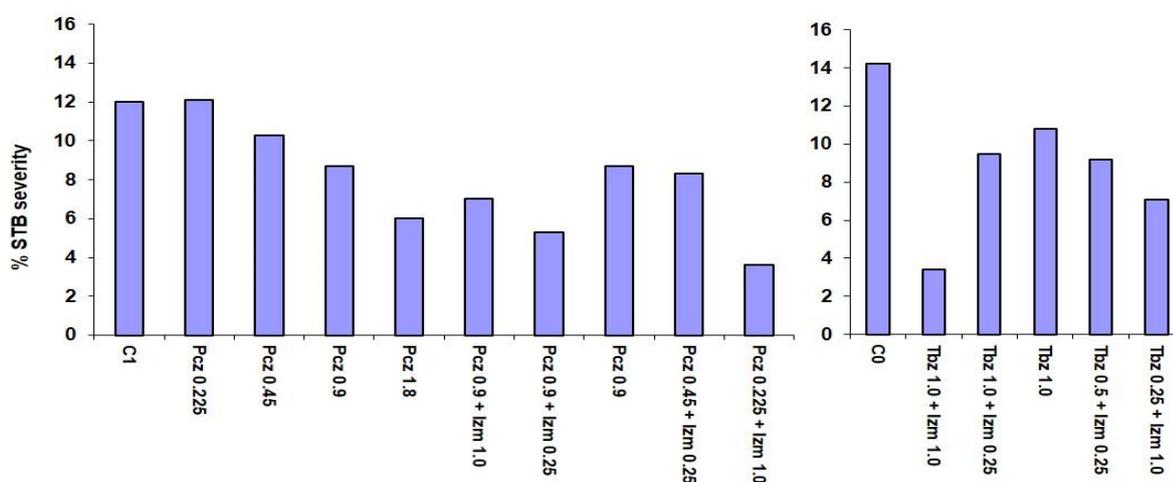
Similar levels of disease control were achieved with a full dose of tebuconazole and prochloraz after the T1 spray. But a later assessment of disease and green leaf area showed that prochloraz based treatment were not so effective in comparison with tebuconazole at GS 75 (Figure 18). Addition of isopyrazam as mixing partner at different doses improved disease control. The only treatment with higher than expected levels of Septoria at T2 was the mixture with both prochloraz and isopyrazam at full dose in comparison with full dose prochloraz with a quarter dose isopyrazam but GLA assessments at GS 75 showed good efficacy for this treatment.

Figure 18. Mean levels of Green Leaf Area (%) on Leaf 2 and Leaf 1 (flag) at location Rosemaund at GS 75 in 2012. Pre-spray (4 weeks prior T1) with tebuconazole (Folicur 1.0 L/ha) was carried out for all prochloraz based treatments and untreated C1 plots (see Table 5 for all treatments applied at T1 and T2).
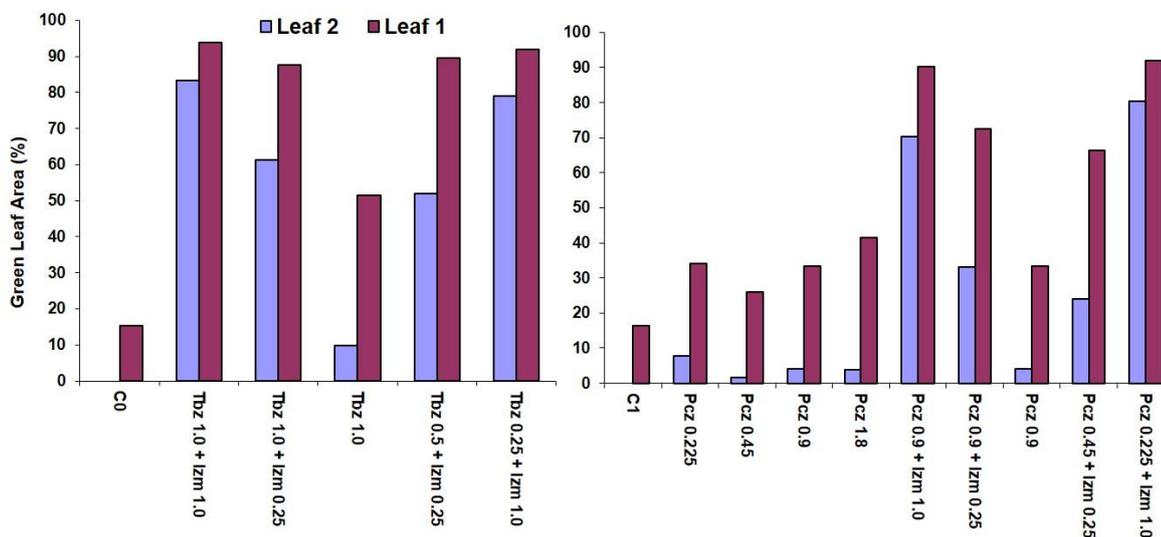

Results of the SNP detection Pyrosequencing assays are shown in Figure 19. The tebuconazole pre-spray reduced the frequency of both V136A and D134G (D134G frequency decreased from 20 to 6 % and V136A frequency from 30 to 10 %) but did not show an effect on the frequency of A379G and I381V alleles. Solo treatments of prochloraz showed that an increasing dose was associated with an increase of D134G and, more clearly, V136A alleles and a decrease of A379A alleles. The effect of the mixture treatments showed also clear patterns. In comparison with a full dose of prochloraz, adding increasing amounts of isopyrazam in mixtures with prochloraz reduced selection for V136A alleles up to 2-fold. Reducing the dose of prochloraz when using similar doses of isopyrazam in mixtures did not result in a significant change of V136A allele frequency. The opposite effects were measured for A379G alleles.

Figure 19. Selection for CYP51 alleles in untreated and fungicide-treated field populations of *Zymoseptoria tritici* sampled 3 weeks post T2 at Rosemaund (Herefordshire) in 2012. Pre-spray with tebuconazole (Folicur 1.0 L/ha) was carried out for all prochloraz based treatments and control plots C1 (see Table 5 for more details on treatments).
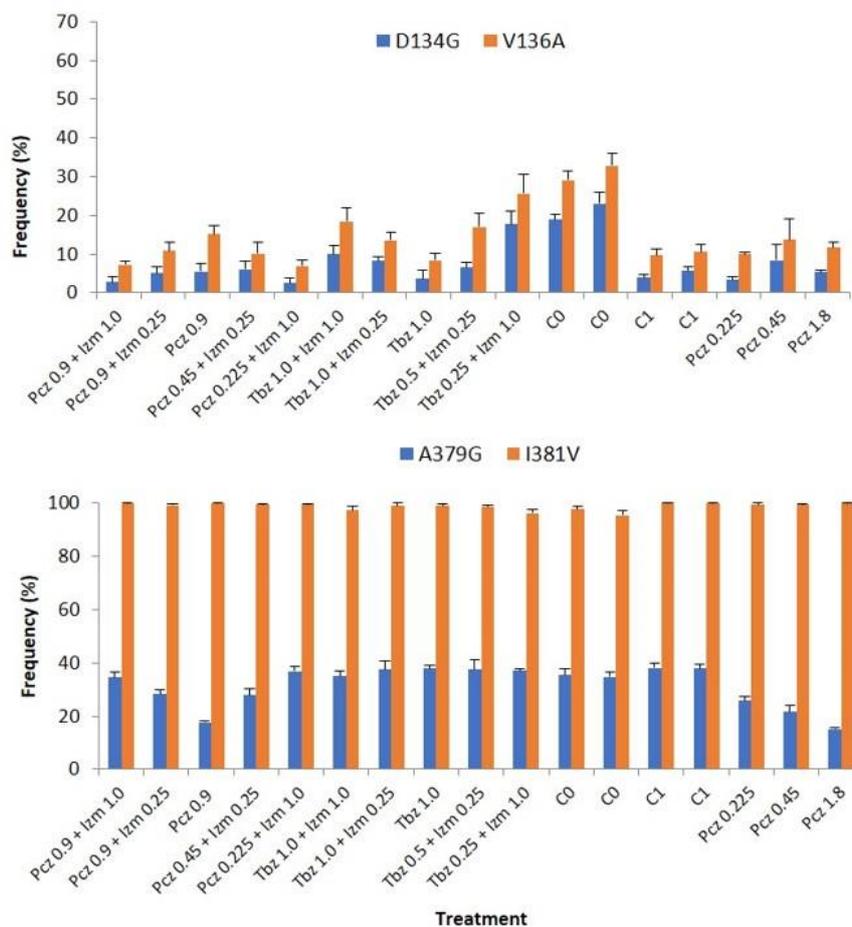
A full dose tebuconazole had no impact on the frequency of A379G and I381V alleles but the frequency D134G and V136A alleles decreased 3 to 4-fold. In comparison with a full dose of tebuconazole, adding increasing amounts of isopyrazam in mixture with tebuconazole reduced selection against D134G and V136A alleles. Reducing the dose of tebuconazole when using similar doses of isopyrazam in mixtures did not show clear patterns of changes in frequencies of both D134G and/or V136A alleles.

The fungicide sensitivity testing results from 2012 are shown in Figure 20. All prochloraz based treatments resulted in a decrease of the most sensitive strains to epoxiconazole (EC_{50} < 0.5 ppm) and prothioconazole-desthio (EC_{50} < 0.05 ppm) but an increase of tebuconazole insensitive strains (EC_{50} > 10 ppm).

Figure 20. Sensitivity of *Z. tritici* isolates to prochloraz, tebuconazole, epoxiconazole and prothioconazole-desthio. Isolates sampled from tebuconazole pre-sprayed plots, left untreated (13), treated twice with full dose prochloraz (3) or treated twice with a mixture of full dose prochloraz and isopyrazam (1)) and from plots without pre-spray, treated twice with tebuconazole full dose (8), twice with mixture of full dose tebuconazole and isopyrazam (6) or left untreated (11) at Rosemaund in 2012.
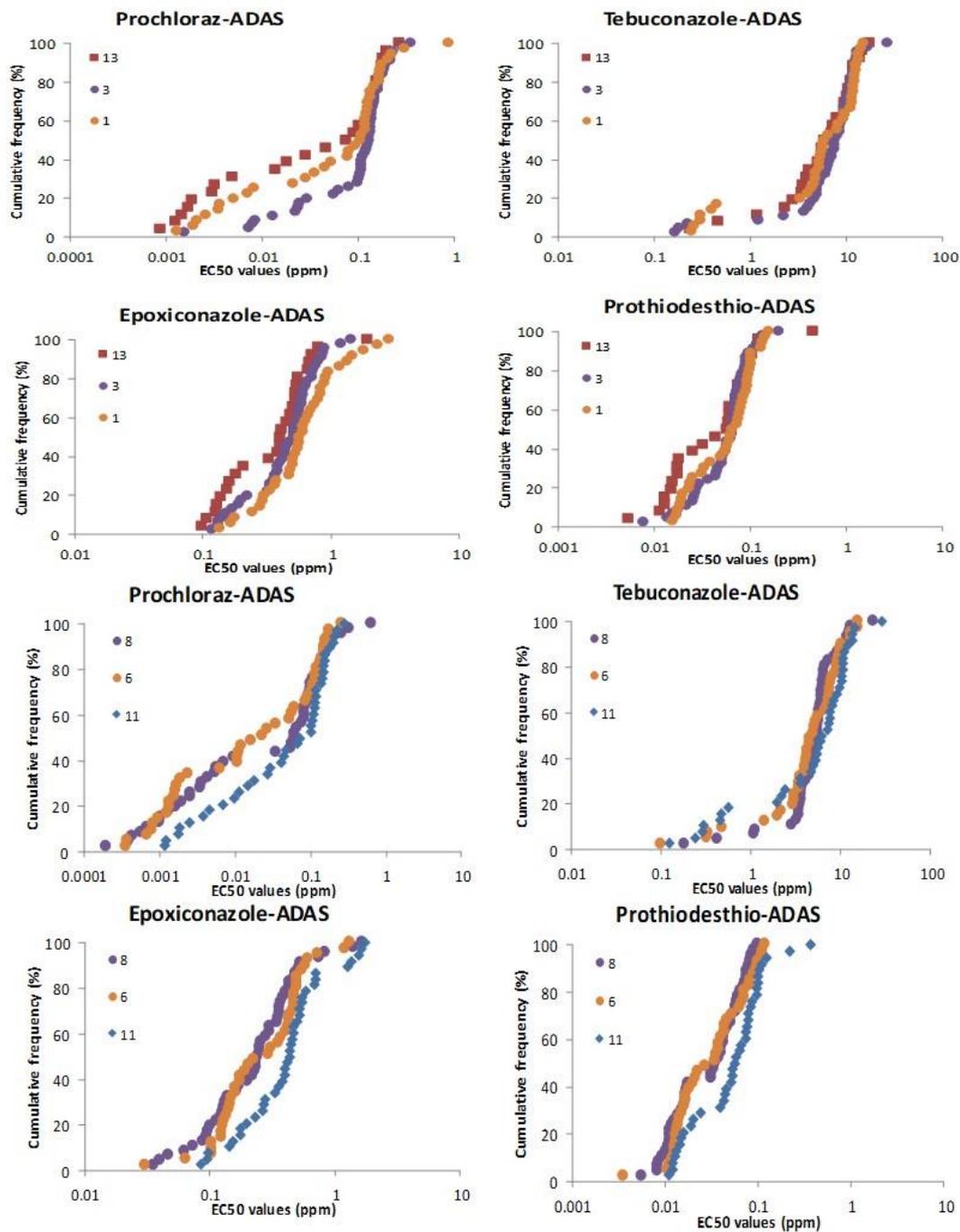
The solo prochloraz treatment resulted in strong selection of prochloraz insensitive strains ($EC_{50} > 0.1$). This selection was less for the mixture of prochloraz with isopyrazam where higher levels of extreme prochloraz sensitive strains ($EC_{50} < 0.01$) remained in the population. All tebuconazole based treatments (solo and mixture with isopyrazam) resulted in an increase of tebuconazole insensitive strains ($EC_{50} > 3$ ppm) and increase of highly prochloraz sensitive strains ($EC_{50} < 0.01$ ppm). All tebuconazole based treatments also selected for epoxiconazole sensitive strains ($EC_{50} < 0.5$ ppm) and prothioconazole-desthio sensitive strains ($EC_{50} < 0.05$ ppm).

2.4.8.2 SRUC Septoria trial at Stanley in 2012

The trial at Stanley was carried out as planned. The crop (cv. Consort) was drilled in September 2011 and received a pre-spray, 4 weeks prior T1, a T1 spray (GS 30 on 16 April 2012) and a T2 spray (GS 37 on 28 May). During the trial, disease levels were recorded at T1, T2 and GS 75. Figure 21 shows the mean levels of Stb on flag leaves recorded at GS 65-69 (16 July).

Figure 21. Mean level of Septoria tritici blotch (Stb) severity (%) on flag leaves at location Stanley (Perthshire) in 2012. Pre-spray (4 weeks prior T1) with tebuconazole (Folicur 1.0 L/ha) was carried out for all prochloraz based treatments and untreated C1 plots (see Table 5 for all treatments applied at T1 and T2).
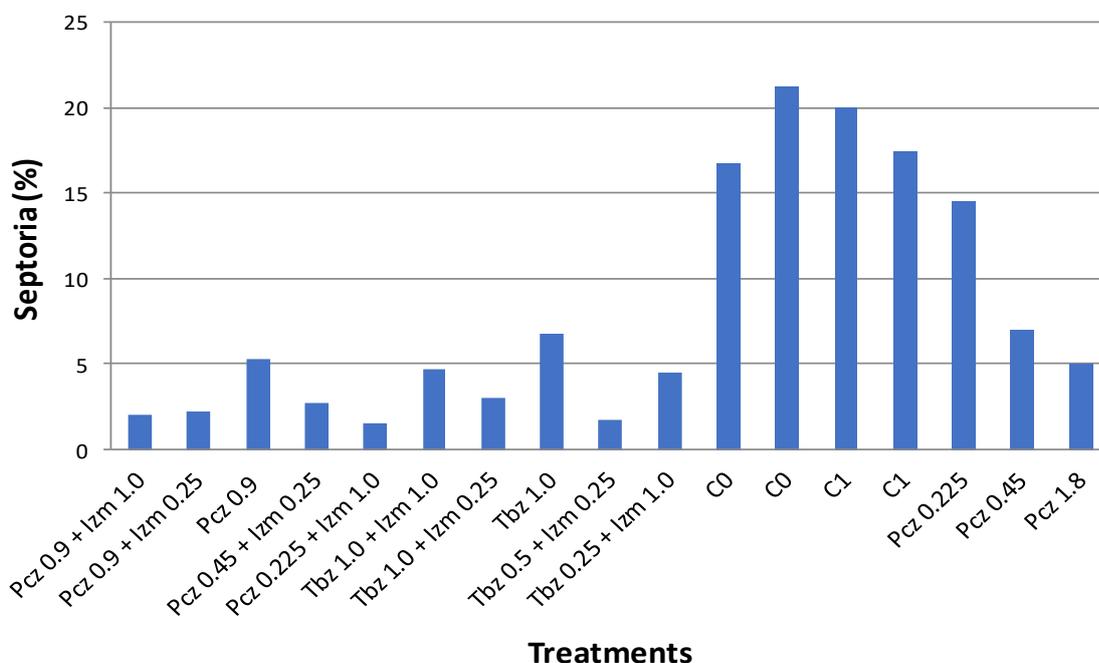
The level of disease control with a full dose of prochloraz was slightly better than that with a full dose of tebuconazole at Stanley. Addition of isopyrazam at different doses as mixing partner with prochloraz improved disease control.

Results of the SNP detection Pyrosequencing assays are shown in Figure 22.

Figure 22. Selection for CYP51 alleles in untreated and fungicide-treated field populations of *Zymoseptoria tritici* sampled 3 weeks post T2 at Stanley (Perthshire) in 2012. Pre-spray with tebuconazole (Folicur 1.0 L/ha) was carried out for all prochloraz based treatments and control plots C1 (see Table 5 for more info on all treatments applied at T1 and T2).
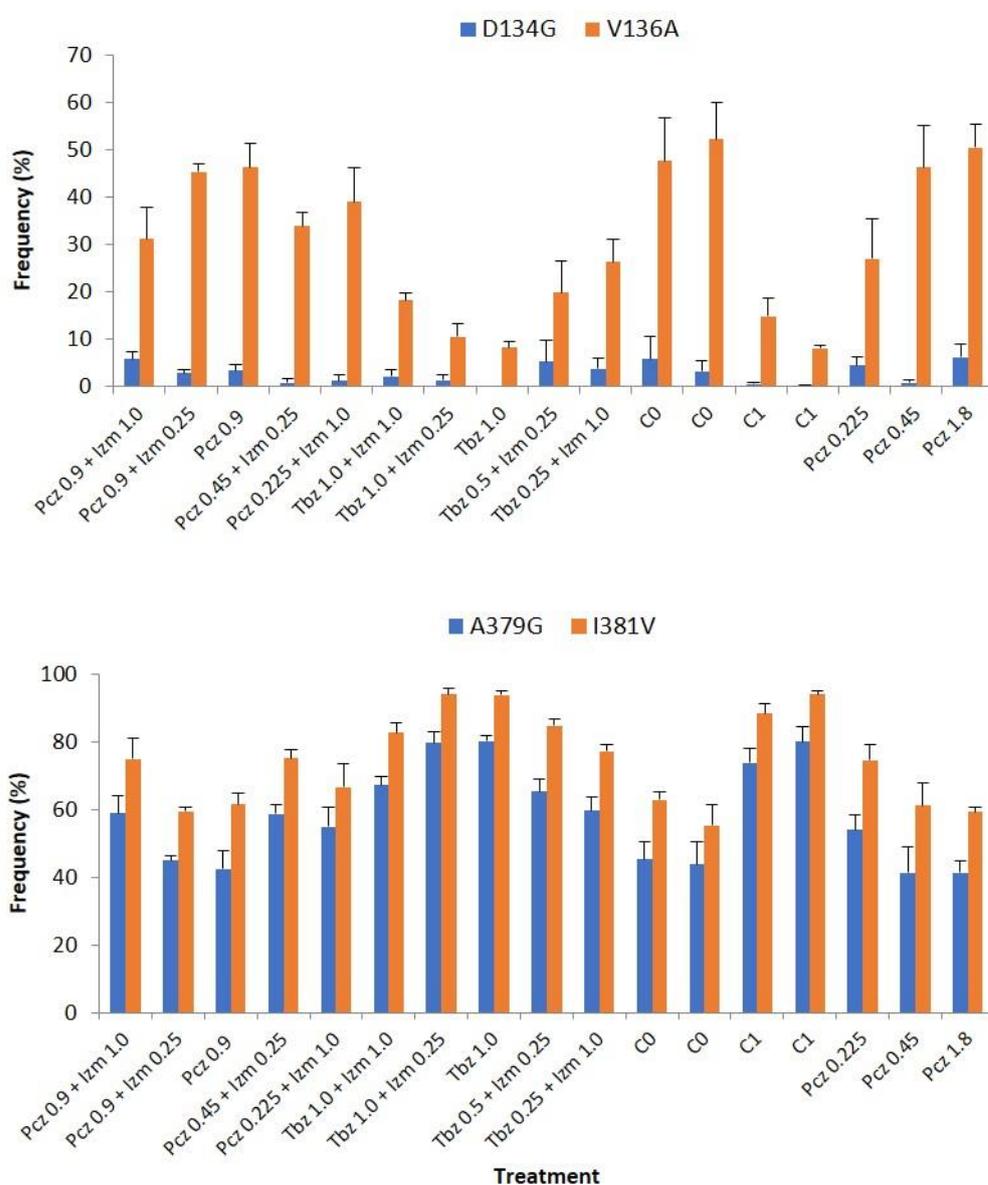
The tebuconazole pre-spray reduced the frequency of V136A from approximately 50 to 10 %. An opposite effect was measured for A379G and I381V alleles with frequencies increasing from 40 to 80 % and 60 to 90 % respectively. The D134G allele frequency was very low, with frequencies below 10% measured for all populations tested. Solo treatments of prochloraz showed that an increasing dose was associated with an increase of V136A alleles and a decrease of A379A and I381V alleles. The effect of the mixture treatments showed also clear patterns. In comparison with a full dose of prochloraz, adding increasing amounts of isopyrazam in mixtures with prochloraz reduced selection for V136A alleles. Reducing the dose of prochloraz in combination with a quarter dose of isopyrazam did further reduce the frequency of V136A alleles but this effect was not measured for mixtures of prochloraz with a full dose of isopyrazam. The opposite effects were measured for A379G and I381V alleles.

A full dose tebuconazole increased the frequency of A379G and I381V alleles from 40 to 80 % and 60 to 90 %, respectively. In addition, the frequency of V136A alleles decreased from 50 to 10 %. In comparison with a full dose of tebuconazole, adding increasing amounts of isopyrazam in mixtures with tebuconazole reduced selection for A379G and I381V alleles and against V136A alleles. Reducing the dose of tebuconazole when using similar doses of isopyrazam did reduce the selection for A379G and I381V alleles and against V136A alleles further.

The fungicide sensitivity test results are shown in Figure 23. All prochloraz based treatments resulted in a strong decrease of the most sensitive strains to epoxiconazole ($EC_{50} < 0.5$ ppm) and prothioconazole-desthio ($EC_{50} < 0.05$ ppm) but a weak increase of tebuconazole sensitive strains ($EC_{50} < 3$ ppm). The solo prochloraz treatment resulted in strong selection of prochloraz insensitive strains ($EC_{50} > 0.1$). This selection was slightly less for the mixture of prochloraz with isopyrazam where more highly prochloraz sensitive strains ($EC_{50} < 0.01$) remained in the population. All tebuconazole based treatments (solo and mixture with isopyrazam) resulted in a sharp increase of prochloraz sensitive strains ($EC_{50} < 0.01$ ppm), epoxiconazole sensitive strains ($EC_{50} < 0.5$ ppm) and prothioconazole-desthio sensitive strains ($EC_{50} < 0.05$ ppm) and selected for highly tebuconazole insensitive isolates ($EC_{50} > 10$ ppm)

Figure 23. Sensitivity of *Zymoseptoria tritici* isolates to prochloraz, tebuconazole, epoxiconazole and prothioconazole-desthio. Isolates sampled from tebuconazole pre-sprayed plots, left untreated (13), treated twice with full dose prochloraz (3) or treated twice with a mixture of full dose prochloraz and isopyrazam (1)) and from plots without pre-spray, treated twice with tebuconazole full dose (8), twice with mixture of full dose tebuconazole and isopyrazam (6) or left untreated (11) at Stanley in 2012.
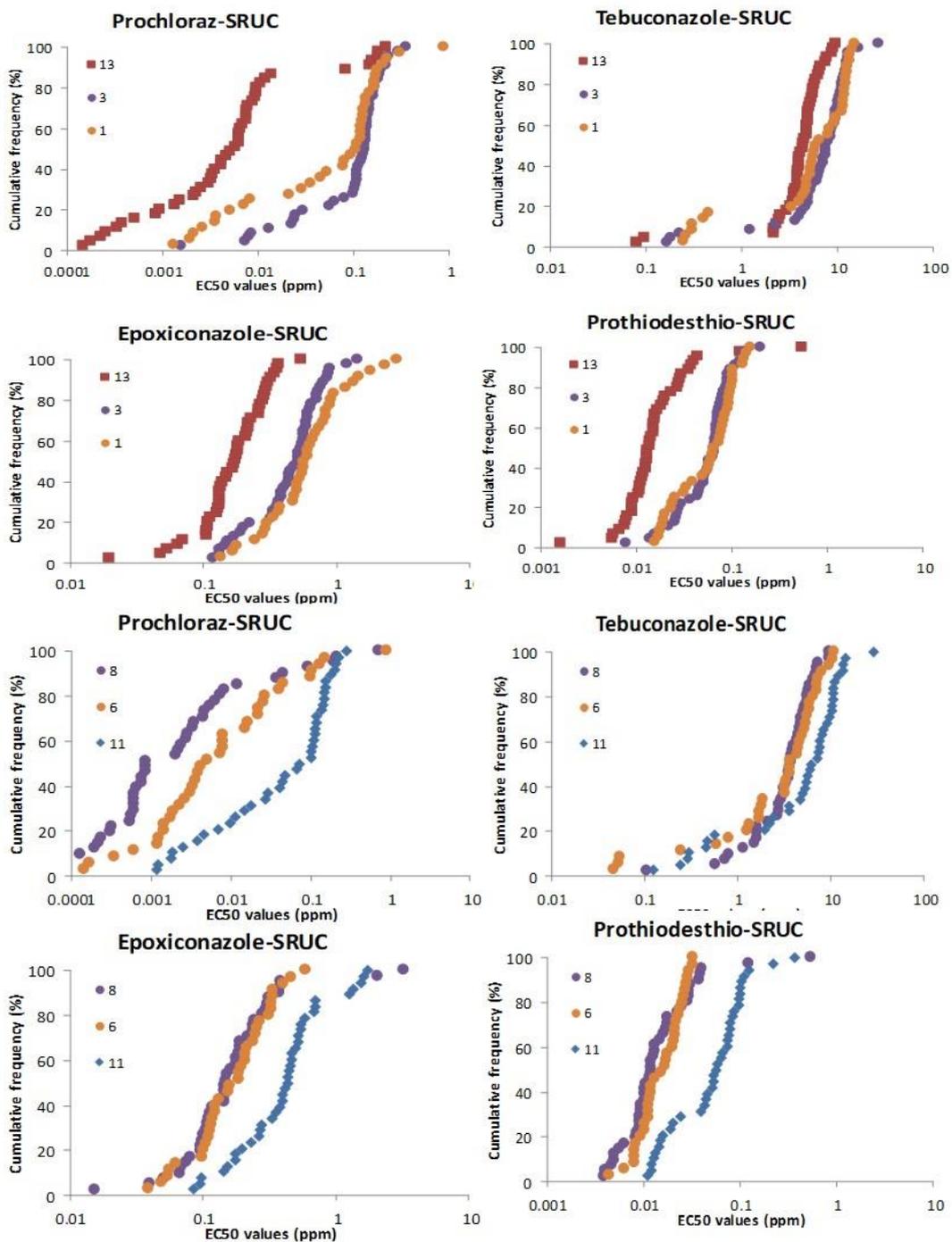
2.4.8.3 Velcourt Septoria trial at Waldershare Park Farms near Dover in 2012

The trial near Dover was carried out in 6 by 2 m plots as planned. The crop (cv. Cordiale) was drilled on 22 September 2011 and received a T0 pre-spray 4 weeks prior T1 (GS 30 22 March 2012), a T1 spray (GS 33 on 1 May) and a T2 spray (GS 37-57 on 30 May). During the trial, disease levels were recorded at T1, T2 and GS 73-75. Figure 24 shows the mean levels of Stb on flag leaves recorded at GS 65-69 (16 July).

Figure 24. Mean level of Septoria tritici blotch (Stb) severity (%) on flag leaves at the Velcourt trial location near Dover in 2012. Visual assessments were done at grain fill GS73-75 on 4th of July. Pre-spray with Folicur (1.0 L/ha) was carried out for all prochloraz based treatments and untreated (C1) four weeks prior to the T1 spray timing (see Table 5 for more details on treatments (T1 + T2 applications).
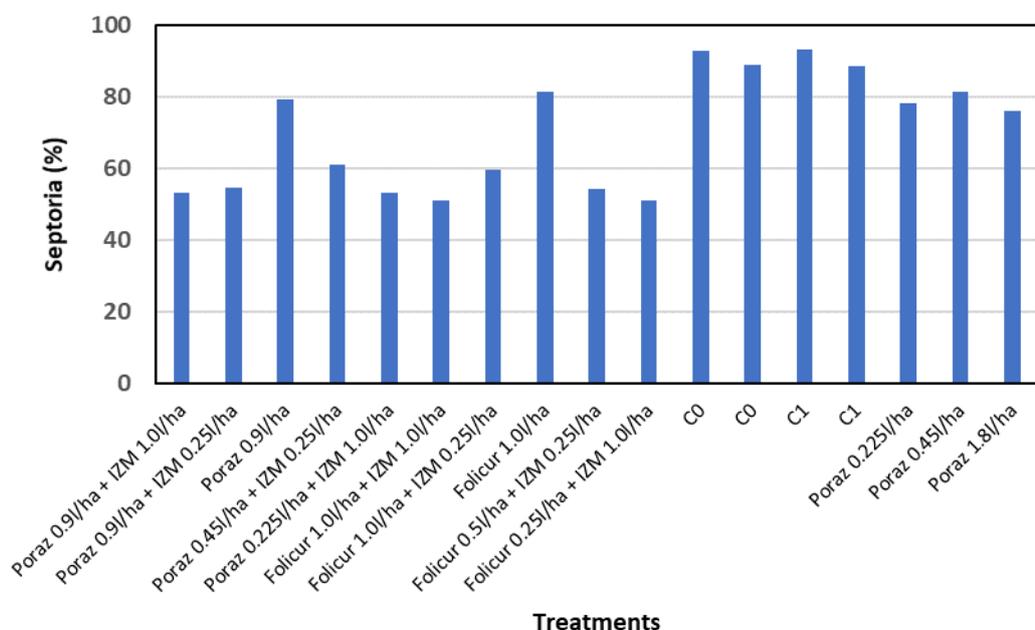
Similar, low levels of disease control were achieved with a full dose of tebuconazole and prochloraz. Addition of isopyrazam at different doses as mixing partner with prochloraz and tebuconazole improved disease control.

Results of the SNP detection Pyrosequencing assays are shown in Figure 25. The tebuconazole pre-spray reduced the frequency of both V136A and D134G (D134G frequency decreased from 15 to 8 % and V136A frequency from 30 to 5 %) but did not show an effect on the frequency of A379G and I381V alleles, where frequencies of 20 and 100%, respectively, were measured.

Figure 25. Selection for CYP51 alleles in untreated and fungicide-treated field populations of *Zymoseptoria tritici* sampled 3 weeks post T2 near Dover (Kent) in 2012. Pre-spray with tebuconazole (Folicur 1.0 L/ha) was carried out for all prochloraz based treatments and control plots C1 (see Table 5 for more details on treatments).
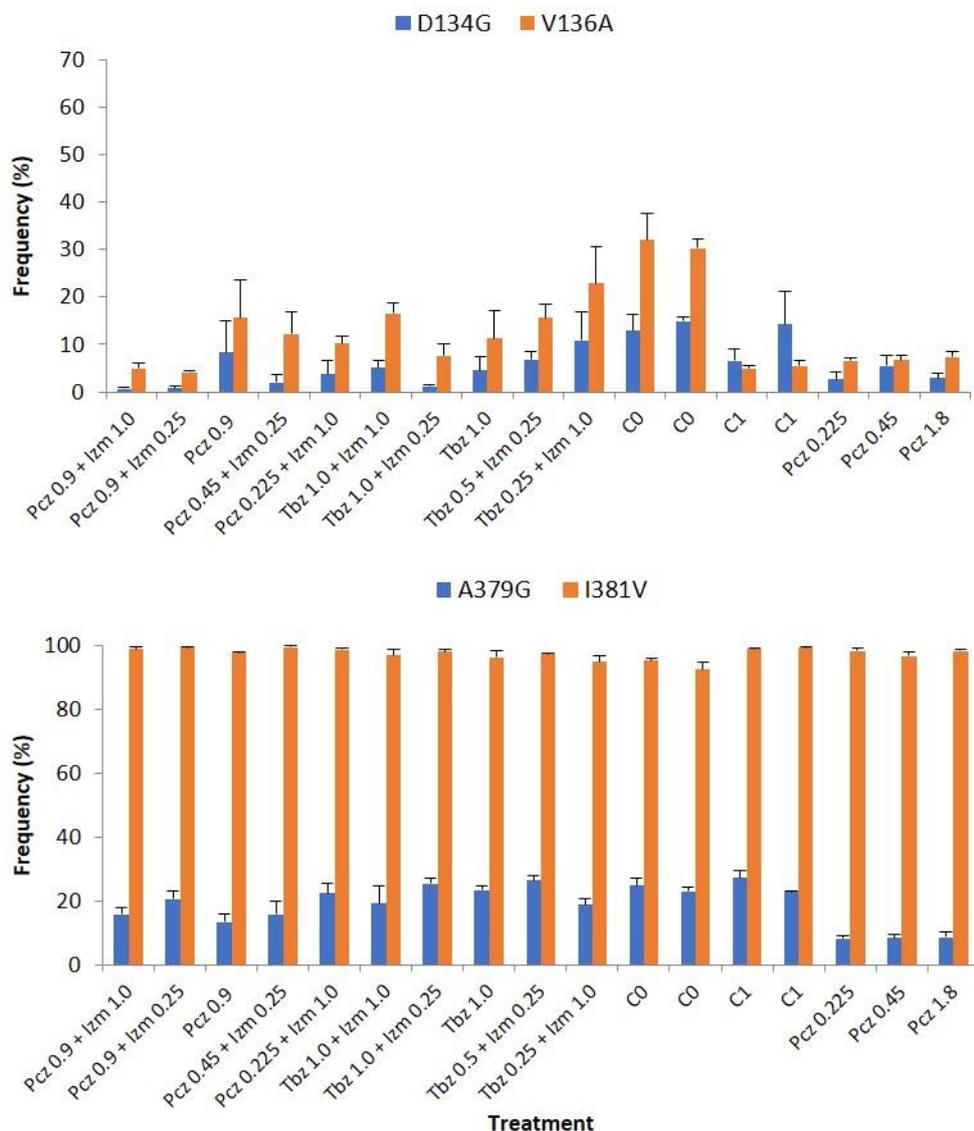
Solo treatments of prochloraz showed no clear selection for alleles, the frequency of V136A alleles was only increased at full dose (from 8 to 15 %) and A379G allele frequencies were similarly reduced at all doses (from 20 to 10 %). The effect of the mixture treatments showed some patterns. In comparison with a full dose of prochloraz, adding increasing amounts of isopyrazam in mixtures with prochloraz reduced selection for V136A alleles up 2 to 3-fold. Reducing the dose of prochloraz in mixture with a full dose isopyrazam, but not a quarter rate, resulted in a further reduction of V136A allele frequency. The opposite effects were measured for A379G alleles.

A full dose tebuconazole had no impact on the frequency of A379G and I381V alleles but the frequency D134G and V136A alleles decreased 3 to 4-fold. In comparison with a full dose of tebuconazole, adding increasing amounts of isopyrazam in mixtures with tebuconazole generally reduced selection against D134G and V136A alleles. An exception was the mixture with a full dose tebuconazole and a quarter dose isopyrazam. Reducing the dose of tebuconazole when mixed with a full dose isopyrazam further reduced selection against D134G and V136A alleles.

The fungicide sensitivity testing results are shown in Figure 26. All prochloraz based treatments resulted in a very weak decrease of the most sensitive strains to epoxiconazole (EC_{50} < 0.5 ppm), prothioconazole-desthio (EC_{50} < 0.05 ppm), tebuconazole (EC_{50} < 3 ppm) and prochloraz (EC_{50} < 0.1 ppm). This selection was slightly less for the mixture of prochloraz with isopyrazam where more strains sensitive to prochloraz, epoxiconazole and prothioconazole, but not tebuconazole, remained in the population. All tebuconazole based treatments (solo and mixture with isopyrazam) resulted only in a clear decrease in tebuconazole sensitive isolates (EC_{50} < 5 ppm).

2.4.9 Bixafen sensitivity testing of strains isolated from the core field trials at Rosemaund, Stanley and Dover in 2012.

In addition of azole sensitivity testing, the bixafen sensitivity of isolates was also measured for all strains isolated from untreated plots and after various treatments 3-4 weeks after the T2 spray application. The results of the bixafen sensitivity testing are displayed in Figure 27. Overall, there were no significant changes or sensitivity shifts detected. Most of the isolates had EC_{50} values between 0.01 and 1.0 µm ml^{-1} (ppm). No alterations of SdhB, C and D were found in a selection of less sensitive strains (EC_{50} > 0.4 ppm) and other mechanisms such as overexpression of efflux pumps are likely present.

Figure 26. Sensitivity of *Zymoseptoria tritici* isolates to prochloraz, tebuconazole, epoxiconazole and prothioconazole-desthio. Isolates sampled from tebuconazole pre-sprayed plots, left untreated (13), treated twice with full dose prochloraz (3) or treated twice with a mixture of full dose prochloraz and isopyrazam (1)) and from plots without pre-spray, treated twice with tebuconazole full dose (8), twice with mixture of full dose tebuconazole and isopyrazam (6) or left untreated (11)) near Dover in 2012.
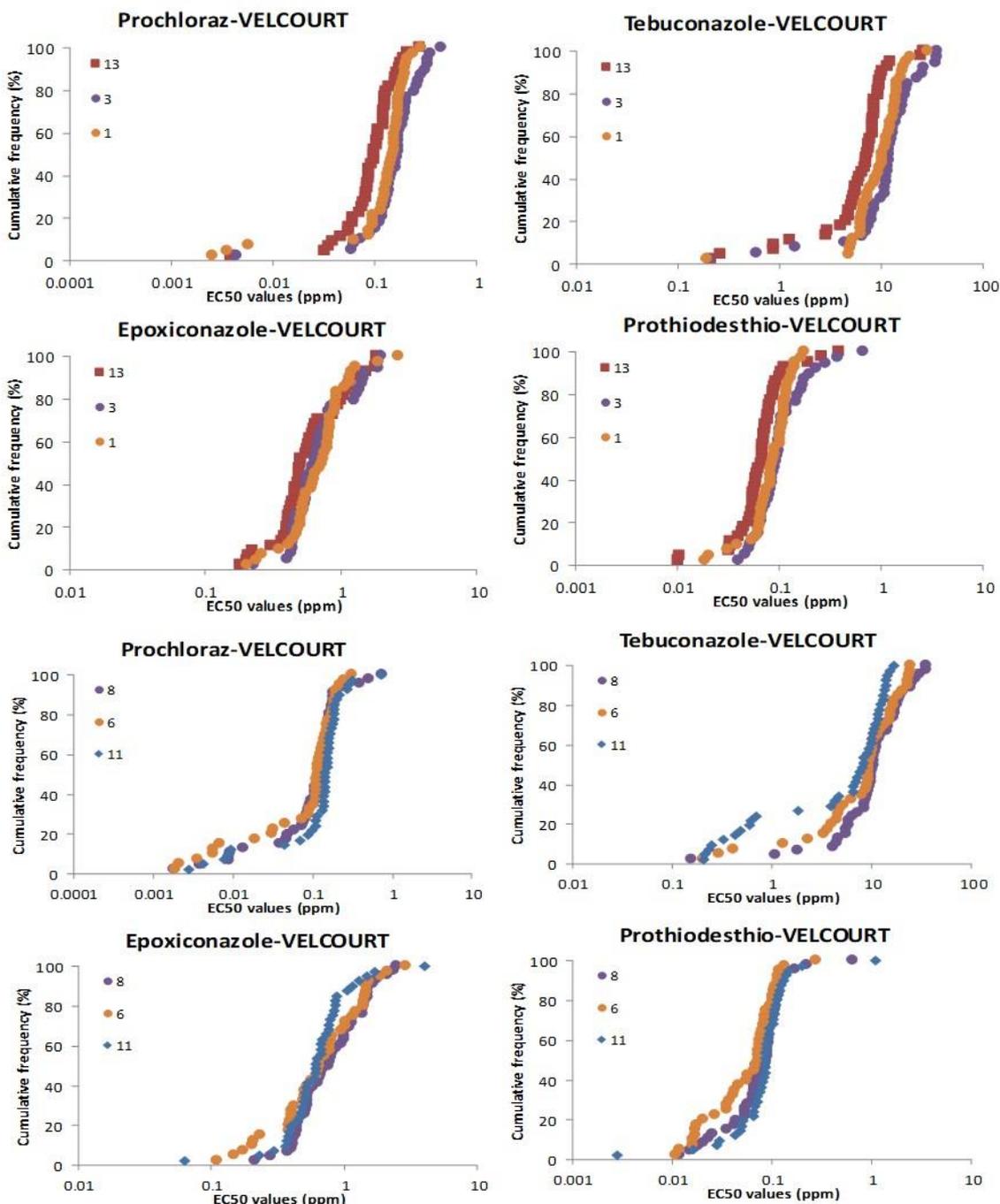
Figure 27. Sensitivity of *Zymoseptoria tritici* isolates to bixafen. Isolates sampled from tebuconazole pre-sprayed plots, left untreated (13), treated twice with full dose prochloraz (3) or treated twice with a mixture of full dose prochloraz and isopyrazam (1)) and from plots without pre-spray, treated twice with tebuconazole full dose (8), twice with mixture of full dose tebuconazole and isopyrazam (6) or left untreated (11) at Rosemaund (ADAS), Stanley (SRUC) and Dover (Velcourt) .
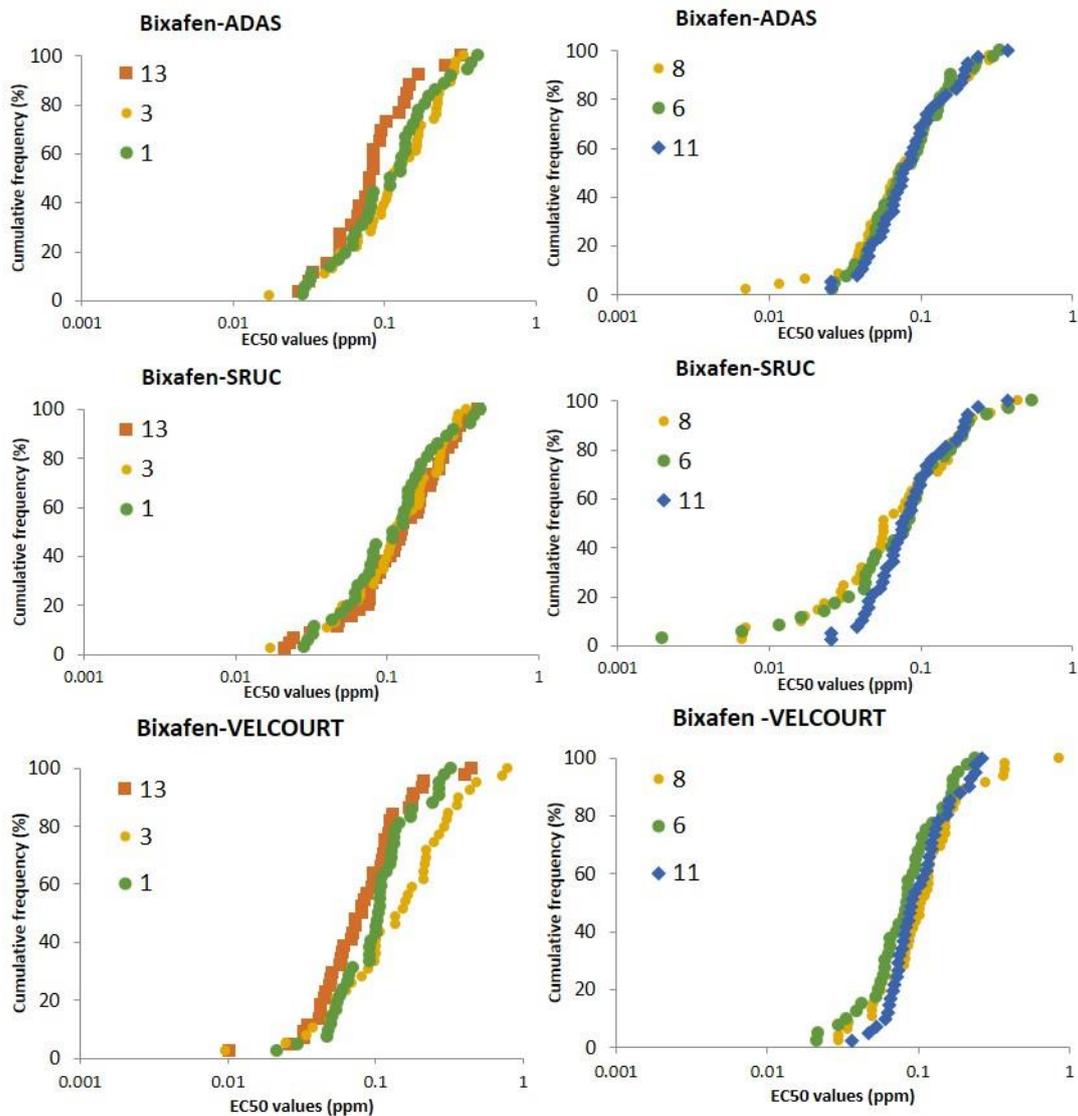
2.4.10 Characterisation of *Z. tritici* strains from the core field trial at Stanley in 2012.

To understand which isolates are being selected after specific treatments, a selection of strains from the SRUC core field trial were further characterised. The CYP51 sequences from strains isolated from untreated (treatment 11), full dose tebuconazole treated (treatment 8) and full dose tebuconazole with isopyrazam mixture treated plots (treatment 6) (see Figure 23) were determined (see Table 12).

Table 12. Azole phenotype (EC$_{50}$ values in µg ml^{-1}) to CYP51 genotype relationship of *Z. tritici* strains. Strains ranked per treatment according to prochloraz sensitivity

| Strains	Tebuconazole	Epoxiconazole	Prochloraz	Prothio-desthio	Bixafen	CYP51 variant
Untreated plots						
33.18	2.89	0.0674	0.000318	0.0128	0.0969	L50S, S188N, A379G, I381V, Δ, N513K
2.13	4.43	0.118	0.00049	0.013	0.0409	L50S, S188N, A379G, I381V, Δ, N513K
43.18	6.29	0.256	0.000758	0.0186	0.0992	A379G, I381V, Δ, N513K
33.5	5.74	0.26	0.00171	0.0241	0.0533	L50S, S188N, A379G, I381V, Δ, N513K
33.8	0.615	0.0503	0.00689	0.0105	0.0597	D107V, I381V, N513K, S524T
43.11	1.97	0.092	0.0103	0.0144	0.0276	L50S, I381V, Y461H
33.7	1.74	0.0696	0.0108	0.0125	0.0684	L50S, I381V, Y461H
43.12	8.15	0.816	0.0312	0.0215	0.177	L50S, S188N, A379G, I381V, Δ, N513K
2.12	0.0393	0.03	0.0517	0.00683	0.0134	L50S, V136A, Y461S, S524T
23.4	0.0643	0.0877	0.159	0.0208	0.0779	L50S, D134G, V136A, Y461H
33.20	0.0859	0.139	0.189	0.0255	0.0832	L50S, V136A, Y461S, S524T
2.2	0.102	0.21	0.215	0.0433	0.0738	L50S, V136A, Y461S, S524T
23.15	0.148	0.373	0.306	0.0602	0.081	L50S, V136A, S188N, Δ, S524T
Tebuconazole treated plots						
37.3	3.02	0.102	0.000305	0.0104	0.241	L50S, S188N, A379G, I381V, Δ, N513K
37.9	6.74	0.255	0.000475	0.00586	0.0655	L50S, S188N, A379G, I381V, Δ, N513K
26.2	3.37	0.197	0.000814	0.0122	0.06	L50S, S188N, I269V, A379G, I381V, Δ, N513K
5.20	5.02	0.312	0.000965	0.0121	0.0661	L50S, S188N, A379G, I381V, Δ, N513K
26.13	5.2	0.209	0.00281	0.0167	0.0672	L50S, S188N, A379G, I381V, Δ, N513K
26.3	4.12	0.374	0.0064	0.0152	0.071	L50S, S188N, A379G, I381V, Δ, N513K
5.2	8.8	0.409	0.00902	0.0236	0.31	L50S, S188N, A379G, I381V, Δ, N513K
37.20	0.432	0.807	0.0137	0.785	0.0342	L50S, V136A, I381V, Y461S, S524T
14.1	2.28	0.308	0.0308	0.0669	0.136	D107V, I381V, Y461H, N513K, S524T
5.19	15.4	0.635	0.0409	0.0389	0.41	L50S, S188N, A379G, I381V, Δ, N513K
5.8	6.04	0.504	0.154	0.0501	0.0809	L50S, S188N, I381V, Δ, N513K
37.2	1.91	1.78	0.182	0.203	0.546	V136A, S188N, A379G, I381V, Δ, S524T
Tebuconazole/isopyrazam mixture treated plots						
19.11	3.76	0.201	0.000343	0.0157	0.106	L50S, S188N, A379G, I381V, Δ, N513K
6.16	6.49	0.198	0.000535	0.0221	0.0532	L50S, S188N, A379G, I381V, Δ, N513K
30.13	4.86	0.231	0.000721	0.0125	0.0489	L50S, S188N, A379G, I381V, Δ, N513K
19.9	3.34	0.172	0.000748	0.0128	0.131	L50S, S188N, A379G, I381V, Δ, N513K
6.4	6.24	0.178	0.00178	0.00909	0.0564	L50S, S188N, A379G, I381V, Δ, N513K
30.1	4.18	0.0771	0.00507	0.0125	0.0156	A379G, I381V, Δ, N513K
39.18	1.86	0.108	0.0114	0.00778	0.0337	L50S, I381V, Y461H
6.6	3.23	0.137	0.0204	0.0139	0.0397	L50S, S188N, I381V, Δ, N513K
39.14	0.802	0.116	0.0372	0.00499	0.0654	L50S, V136C, Y461S
39.3	0.909	0.128	0.0452	0.00538	0.0724	L50S, V136C, S188N, Δ, N513K
30.15	1.41	0.159	0.145	0.0057	0.127	L50S, V136C, Y461S
19.1	0.363	1.43	0.149	0.243	0.0884	L50S, V136A, I381V, Y461S, S524T
19.15	0.389	0.787	0.156	0.105	0.149	L50S, D134G, V136A, I381V, Y461H
6.12	0.0842	0.208	0.164	0.0362	0.0319	L50S, V136A, S188N, Δ, N513K, S524T
30.10	0.0704	0.189	0.19	0.023	0.154	L50S, V136A, Y461S, S524T

Isolates of each treatment were selected to cover a wide range of prochloraz sensitivity. Tebuconazole based treatments selected for insensitivity to tebuconazole but the sensitivity to epoxiconazole, prothioconazole-desthio and, particularly, prochloraz increased (Figure 21). The strains from untreated plots showed that 17 out of the 18 strains highly sensitive to prochloraz (EC_{50} < 0.01 ppm) carried A379G alleles, with variant [L50S, S188N, A379G, I381V, Δ, N513K] represented by 14 strains most common. None of the 18 highly prochloraz sensitive strains carried V136A alleles. Of the 11 highly prochloraz insensitive strains, 9 carried a V136A alteration and only one strain carried an A379G alteration, but this was together with a V136A alteration (variant [V136A, S188N, A379G, I381V, Δ, S524T]). Four out of the 11 variants with V136A alleles also carried I381V alleles, with one variant carrying D134G as well ([L50S, D134G, V136A, I381V, Y461H]). The low frequency of D134G detected in field populations was confirmed because D134G was only detected in one other isolate ([L50S, D134G, V136A, Y461H]). S524T was detected in 11 isolates, with 9 of them carrying V136A. Complex CYP51 variants with S524T are often highly insensitive to epoxiconazole and prothioconazole-desthio. Solo tebuconazole applications selected strongly for I381V alleles and the two prochloraz insensitive strains (EC_{50} > 0.1 ppm) were also insensitive to tebuconazole and carried CYP51 variants [L50S, S188N, I381V, Δ, N513K], a CYP51 overexpressing variant due to the presence of a 120 bp promoter insert (Cools *et al.*, 2012), and [V136A, S188N, A379G, I381V, Δ, S524T], a complex variant harbouring S524T and insensitive to both epoxiconazole and prothioconazole-desthio. The mixture of tebuconazole and isopyrazam selected less for I381V alleles and some moderate tebuconazole insensitive strains, being also moderately insensitive to prochloraz, carried CYP51 variants with V136C alleles (e.g. variants [L50S, V136C, Y461S] and [L50S, V136C, S188N, Δ, N513K]).

2.4.11 Core field trial design for the 2012/13 growing season

For the 2012/13 growing season, experiments were repeated using mixture effects with tebuconazole and isopyrazam because of the excellent results from the previous season. Treatments with prochloraz were replaced by epoxiconazole because of available data sets on prochloraz covering two seasons and to obtain results that are closer to what is used in practice. An early season fungicide sensitivity test was also carried out to check for differences in the fungicide sensitivity status between the three core field trial locations and Rothamsted in 2013. Results of the early season azole sensitivity monitoring are shown in Figure 28.

Figure 28. Early season azole sensitivity testing of *Zymoseptoria tritici* populations sampled at Stanley (Perthshire), Rosemaund (Herefordshire), Dover (Kent) and Harpenden (Hertfordshire). Leaf samples were collected pre-T0 from untreated fields.
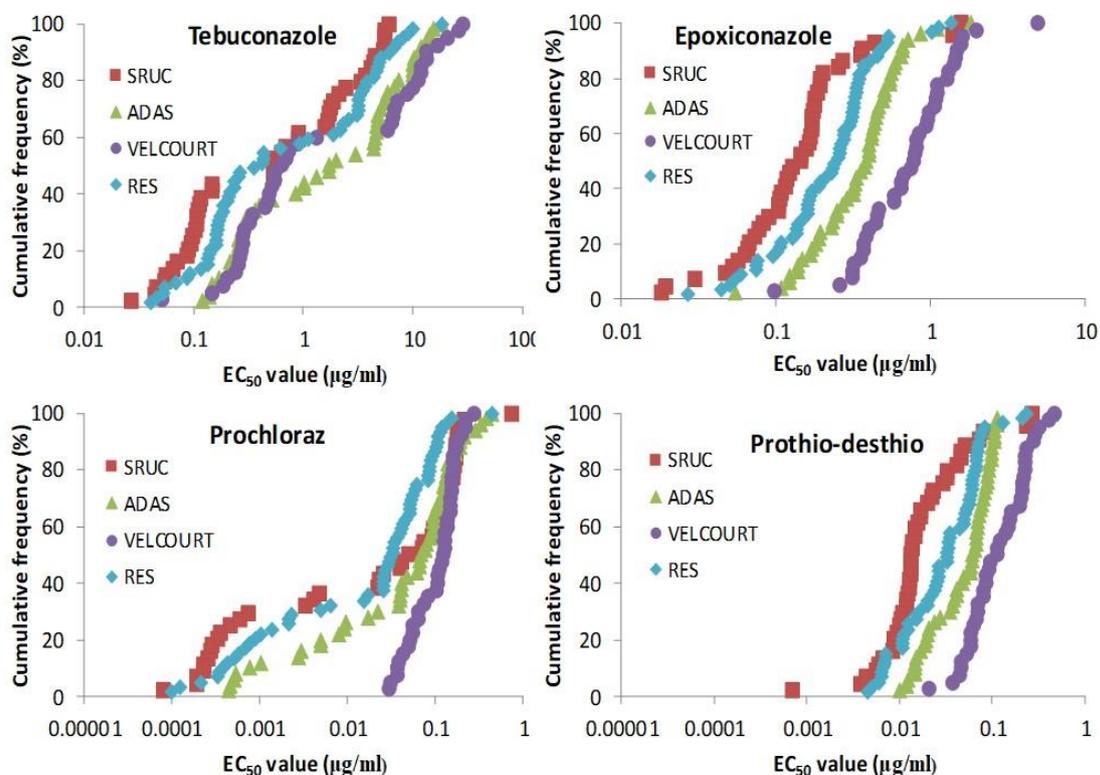
The azole sensitivity profiles for the *Z. tritici* populations sampled in Herefordshire and Hertfordshire were quite similar. The population sampled in Perthshire was in general more sensitive to all azoles tested, particularly to prothioconazole-desthio. The population from Kent was least sensitive to all azoles tested, especially to prochloraz where all isolates had EC_{50} values > 0.01 ppm and to prothioconazole-desthio, where 50 % of the isolates tested showed EC_{50} values > 0.1 ppm.

2.4.11.1 ADAS Septoria trial at Rosemaund in 2013

The trial at Rosemaund was carried out as planned, using 6 by 2 m plots. The crop (cv. Consort) was drilled on 21 September 2012 and received a T1 spray (GS 33 on 1 May) and T2 spray (GS 39-45 on 7 June). During the trial, disease levels were recorded at T1, T2 and GS 75 (16 July). Figure 29 shows the mean levels of Septoria tritici blotch on Leaf 3 at GS75.

Figure 29. Mean level of Septoria tritici blotch (STB) severity (%) on different leaf layers at location Rosemaund (Herefordshire) in 2013. Visual assessments were done at GS75 on 16th of July. (see Table 6 for treatments (T1 + T2 applications).
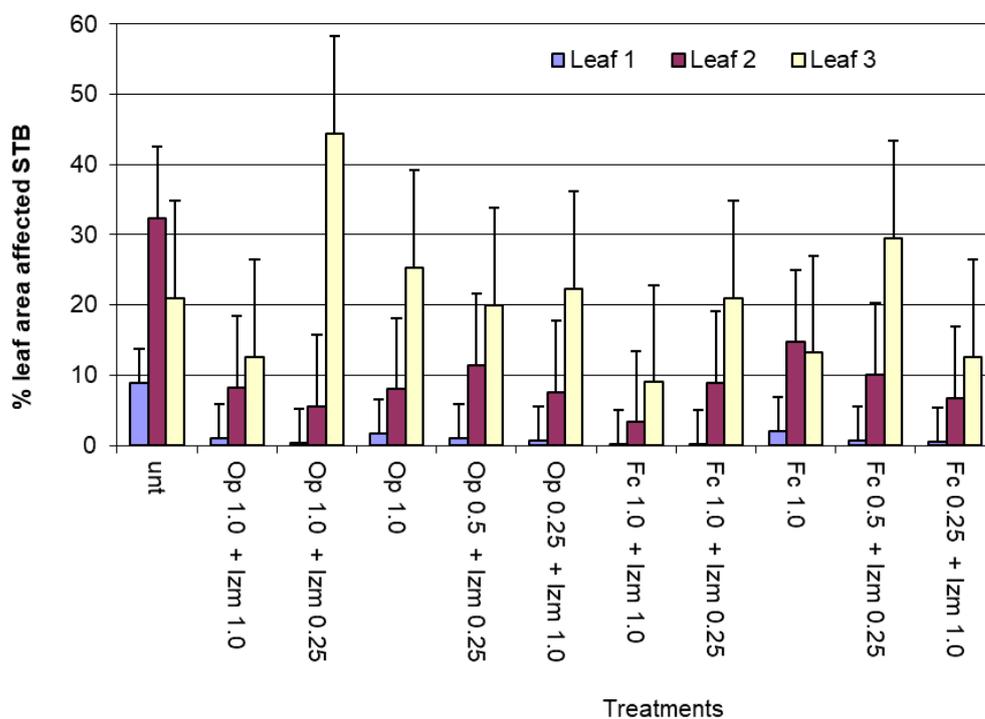
Epoxiconazole and tebuconazole showed a similar efficacy for Septoria leaf blotch control. Isopyrazam in mixture with both tebuconazole and epoxiconazole provided extra control of Septoria leaf blotch on Leaf 1 and Leaf 2.

Results of the SNP detection Pyrosequencing assays are shown in Figure 30. In comparison with untreated plots, the effect of the solo full dose epoxiconazole spray was minimal with only small increases in D134G and V136A allele frequencies from approximately 45 to 50 % and 55 to 65 % respectively. An opposite, but smaller effect was measured for A379G and I381V alleles with frequencies decreasing from 30 to 20 % and 95 to 90 % respectively. These differences in allele frequencies were to small to measure effects of different mixtures based on different doses of both epoxiconazole and isopyrazam.

A full dose tebuconazole increased the frequency of A379G from 30 to 50 % but selection against D134G and V136A was much clearer with frequencies decreasing from 45 to 10 % and 60% to 10 %, respectively. In comparison with a full dose of tebuconazole, adding increasing amounts of isopyrazam in mixtures with tebuconazole reduced selection against D134G and V136A alleles. Reducing the dose of tebuconazole when using similar doses of isopyrazam did reduce the selection against D134G and V136A alleles further.

Figure 30. Detection of CYP51 D134G, V136A, A379A and I381V alleles in untreated and fungicide-treated field populations of *Zymoseptoria tritici* sampled post T2 (GS75) at Rosemaund in 2013. OF, Opus full rate; OH, Opus half rate; OQ, Opus quarter rate; IF, isopyrazam full rate; IQ, isopyrazam quarter rate; FF, Folicur full rate; FH, Folicur half rate; FQ, Folicur quarter rate. (see Table 6 for more details on treatments).
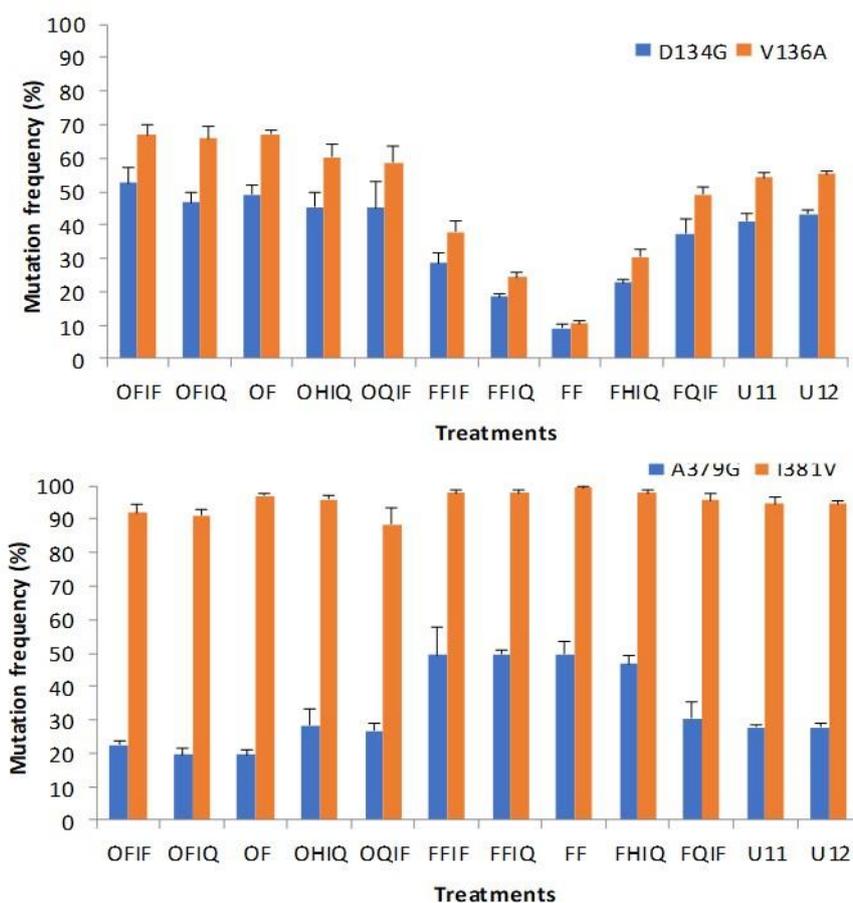
The fungicide sensitivity testing results are shown in Figure 31. Due to few symptoms, not many strains were isolated after treatment with the mixture of Opus (epoxiconazole) and isopyrazam. All epoxiconazole based treatments resulted in an increase of insensitive strains for epoxiconazole ($EC_{50} > 0.5$ ppm), prothioconazole-desthio ($EC_{50} > 0.05$ ppm) and prochloraz ($EC_{50} > 0.1$ ppm), but tebuconazole sensitivity was not affected. The tebuconazole based treatments selected strongly for tebuconazole insensitive strains ($EC_{50} > 3$ ppm) and for prochloraz sensitive strains ($EC_{50} < 0.01$ ppm). The sensitivity to epoxiconazole and prothioconazole-desthio was not affected.

Figure 31. Sensitivity of *Zymoseptoria tritici* isolates to prochloraz, tebuconazole, epoxiconazole and prothioconazole-desthio. Isolates sampled from twice treated plots (T1 + T2) in Rosemaund in 2013. OFIF, mixture Opus full dose and isopyrazam full dose; OF, Opus full dose; FFIF, Folicur full dose and isopyrazam full dose; FF, Folicur full dose; UL, untreated late season (see Table 6 for more info on treatments). Blue arrows show trends in azole sensitivity shifts of strains from treated plots in comparison with strains from untreated plots.
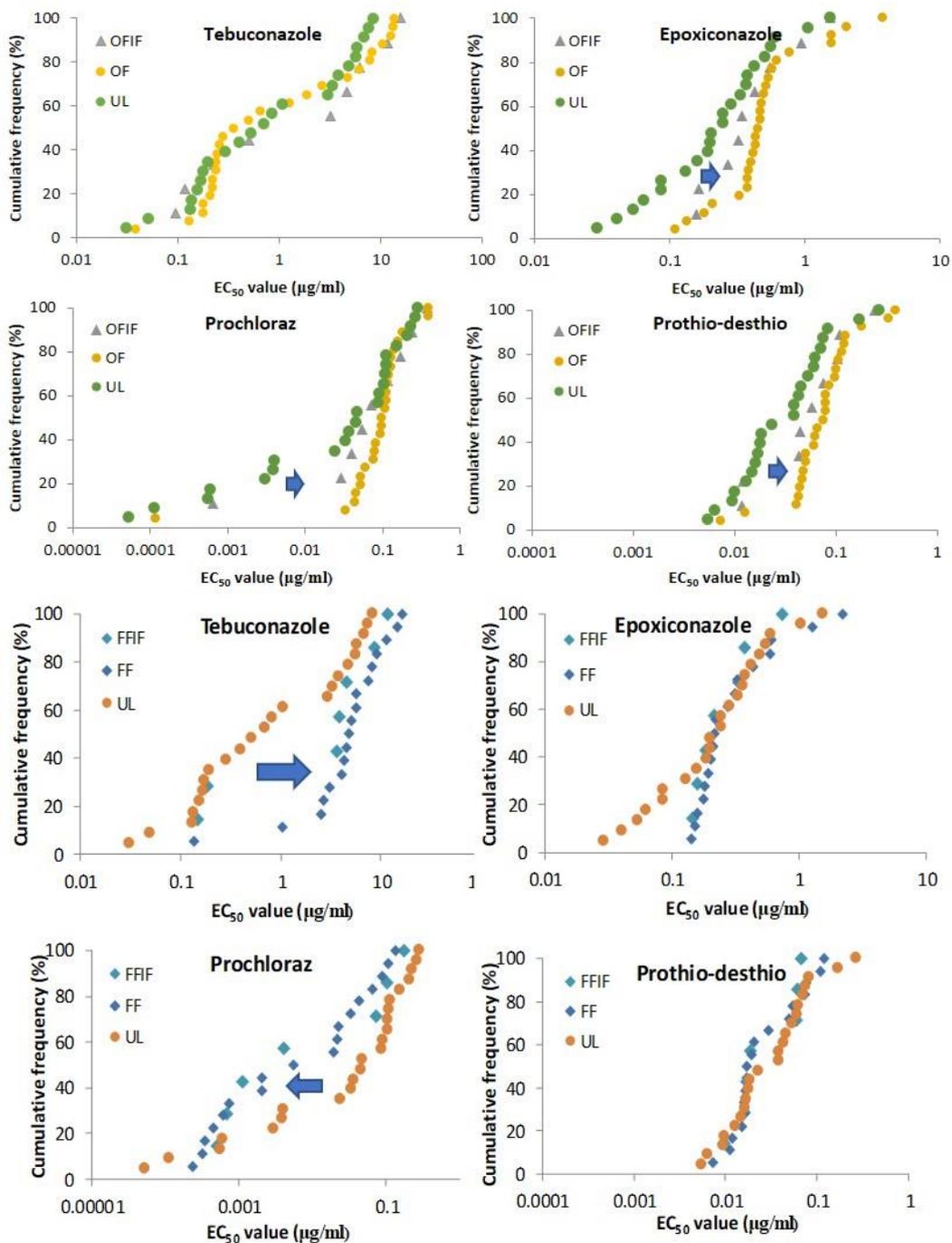
2.4.11.2 SRUC Septoria trial at Stanley 2013

The trial at Stanley was carried out as planned, using 6 by 2 m plots. The crop (cv. Consort) was drilled in September 2012 and received a T1 spray (GS 23-37 on 6 May) and T2 spray (GS 39-45 on 17 June). During the trial, disease levels were recorded at T1, T2 and GS 73-75 (15 July). Figure 32 shows the mean levels of Stb on 4 different leaf layers (Leaf 5 to Leaf 2). Epoxiconazole full dose controlled Stb better than the full dose application of tebuconazole. Adding isopyrazam as mixing partner generally increased the level of disease control, particularly on leaf 3.

Figure 32. Mean level of Septoria tritici blotch (Stb) severity (%) on different leaf layers at location Stanley (Perthshire) in 2013. Visual assessments were done at GS 73-75 on 15th of July. (see Table 6 for more details on treatments (T1 + T2 applications).
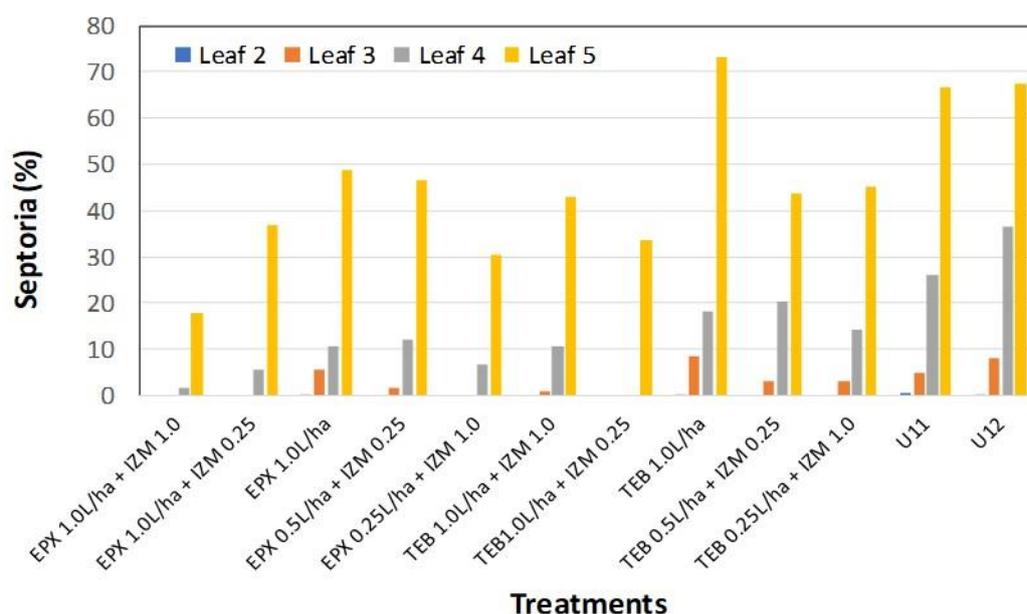
Results of the SNP detection Pyrosequencing assays are shown in Figure 33. In comparison with untreated plots, the solo full dose epoxiconazole sprays selected for D134G, V136A and I381V alleles, with frequencies increasing from 10 to 20 %, 50 to 70 % and 65 to 75 %, respectively. The frequency of A379G decreased from 50 to 30 %. In comparison with a full dose of epoxiconazole, adding increasing amounts of isopyrazam in mixtures with tebuconazole reduced selection for D134G, V136A and I381V and against A379G alleles. Reducing the dose of epoxiconazole when using similar doses of isopyrazam did not reduce the selection for D134G, V136A and I381V alleles further, but selection against A379G alleles decreased.

Figure 33. Selection for CYP51 D134G, V136A, A379A and I381V in untreated and fungicide-treated field populations of *Zymoseptoria tritici* sampled post-T2 (GS73) at Stanley (Perthshire) in 2013. Allele frequencies were measured using SNP detection Pyrosequencing assays (see Table 6 for more details on treatments).
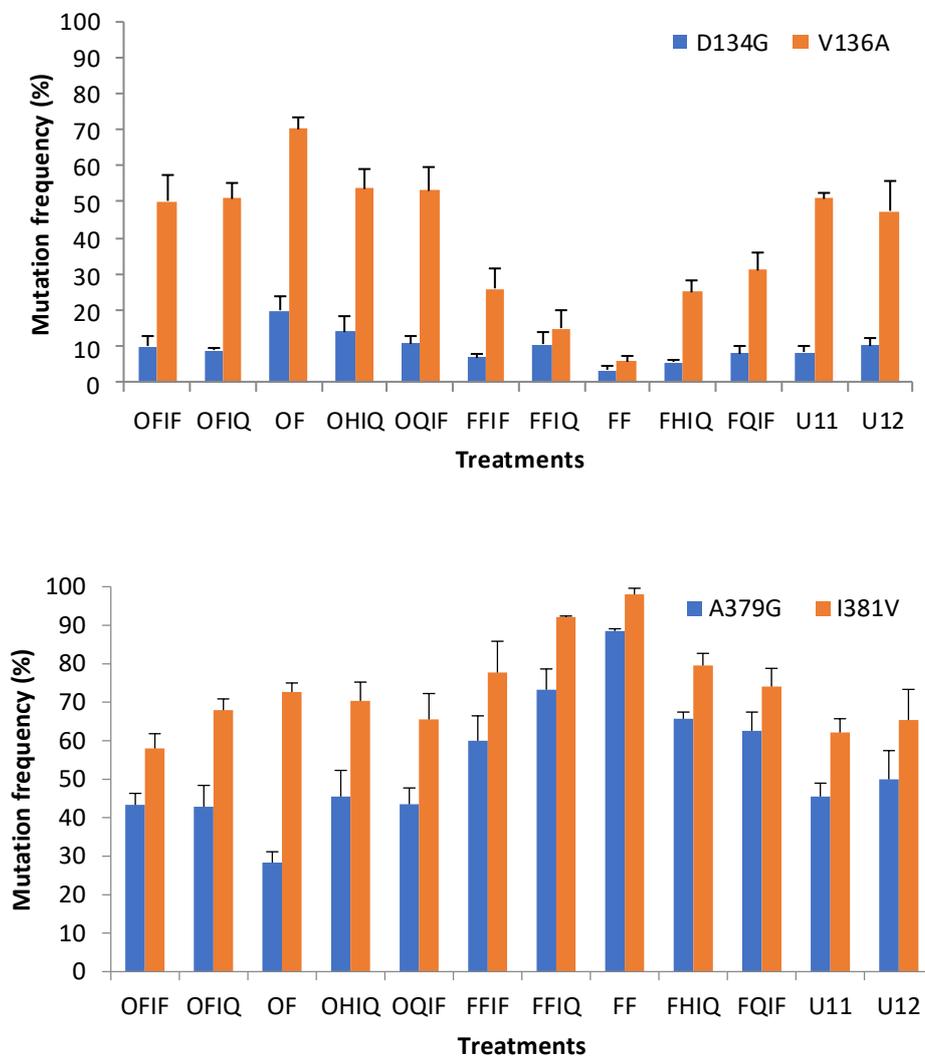
Full dose tebuconazole treatments, applied at T1 and T2, increased the frequency of A379G and I381V alleles from 50 to 90 % and 65 to 100 %, respectively. It selected against D134G and V136A alleles, with frequencies decreasing from 10 to 5 % and 50 to 5 %, respectively. In comparison with full dose of tebuconazole, adding increasing amounts of isopyrazam in mixtures with tebuconazole clearly reduced selection against D134G and V136A alleles and for A379G and I381V alleles. Reducing the dose of tebuconazole when using similar doses of isopyrazam did reduce the selection for all alleles.

The fungicide sensitivity testing results are shown in Figure 34.

Figure 34. Sensitivity of *Zymoseptoria tritici* isolates to prochloraz, tebuconazole, epoxiconazole and prothioconazole-desthio. Isolates sampled from twice treated plots (T1 + T2) in Stanley in 2013. OFIF, mixture Opus full dose and isopyrazam full dose; OF, Opus full dose; FFIF, Folicur full dose and isopyrazam full dose; FF, Folicur full dose; UL, untreated late season (see Table 6 for more info on treatments). Blue arrows show trends in azole sensitivity shifts of strains from treated plots in comparison with strains from untreated plots.
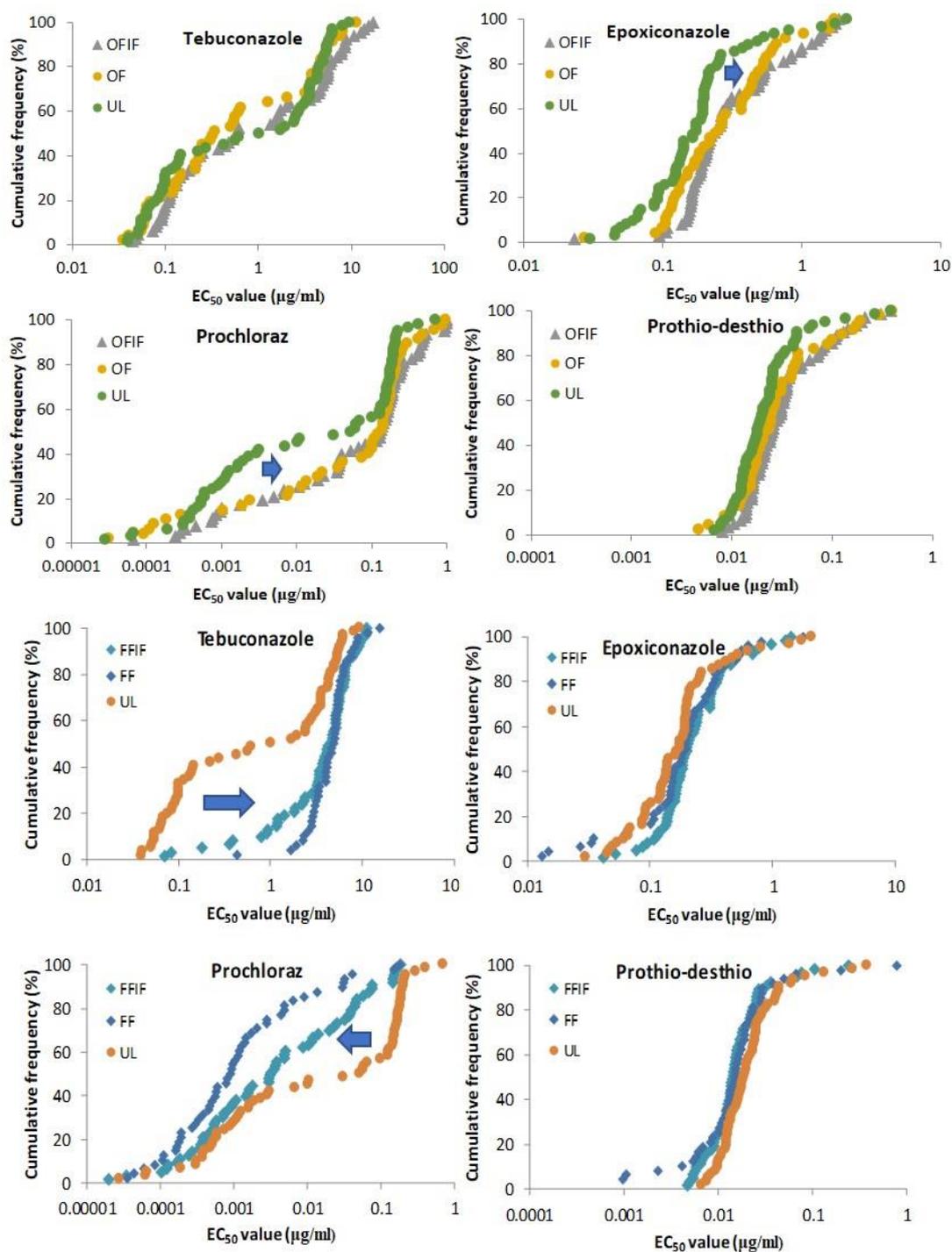
All epoxiconazole based treatments resulted in an increase of insensitive strains for epoxiconazole ($EC_{50} > 0.5$ ppm) and prochloraz ($EC_{50} > 0.1$ ppm), but the prothioconazole-desthio and tebuconazole sensitivity was not affected. The tebuconazole based treatments selected strongly for tebuconazole insensitive strains ($EC_{50} > 3$ ppm) and for prochloraz sensitive strains ($EC_{50} < 0.01$ ppm), while the sensitivity to epoxiconazole and prothioconazole-desthio was not affected. This selection for tebuconazole insensitive strains and prochloraz sensitive strains was less pronounced when isopyrazam at full dose was added as mixing partner.

2.4.11.3 Velcourt Septoria trial at Dover in 2013

The trial at Martin Lodge near Dover was carried out as planned, using 6 by 2 m plots. The crop (cv. Solstice) was drilled on 10 September 2012 and received a T1 spray (GS 32 on 8 May 2013) and T2 spray (GS 37-45 on 29 May). During the trial, Septoria disease levels were recorded at T1, T2 and at mid grain fill (4 July). Figure 35 shows the mean Stb levels on 3 different leaf layers (Leaf 1 to Leaf 3).

Figure 35. Mean level of Septoria tritici blotch (Stb) severity (%) on different leaf layers at Dover in 2013. Visual assessments were done at mid grain fill on 4th of July. (see Table 6 for more details on treatments (T1 + T2 applications).
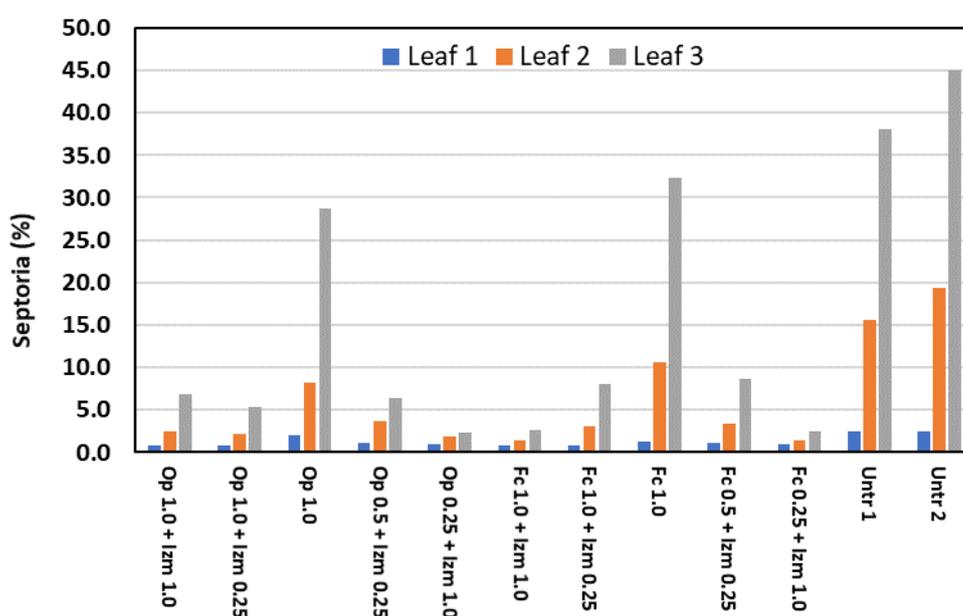
Full doses of epoxiconazole and tebuconazole applied at T1 and T2 showed a similar efficacy for Septoria leaf blotch control. Adding isopyrazam as mixing partner greatly increased the level of disease control for all three leaf layers. Unfortunately, the leaves sent in for DNA extraction and isolation of strains didn't contain much pycnidia

and were mouldy so that, unfortunately, isolations and SNP detection Pyrosequencing assays could not be carried out.

2.4.12 Fungicide Performance trials 2013-2018

Leaves with Septoria symptoms were sampled each year at locations where the Fungicide Performance against Septoria was measured. The locations with Septoria trials during 2014-2018 were: Fife (Scotland) run by SRUC, Sutton Scotney (Hampshire) (NIAB-TAG), Rosemaund (Herefordshire) (ADAS), Cardigan (Wales) (ADAS) and Carlow (Ireland) Teagasc. Samples (50 leaves with Septoria symptoms) were taken at the start of the season before spraying (March-April) and 3-4 weeks after a single T1 or T2 spray application (20 leaves with symptoms per replicate plot (3 or 4 replicate plots were sampled in each trial)) with a full dose of an azole (epoxiconazole (Opus Max, Ignite or Bassoon)) or the SDHI fluxapyroxad (Imtrex)).

2.4.12.1 Azole and SDHI sensitivity shifts in *Zymoseptoria tritici* field populations sampled from the AHDB Fungicide Performance trials during 2013-2018

Figures 36 and 37 show the epoxiconazole sensitivity profiles from isolates sampled from fungicide treated (single spray of full dose Ignite (epoxiconazole) or Imtrex (fluxapyroxad)) and untreated plots at Rosemaund and Sutton Scotney, respectively, during 2013-2018.

As expected, in comparison with the untreated populations, Ignite selected for epoxiconazole insensitivity with the largest shifts for the Rosemaund populations recorded in 2013 and 2015 (Figure 36). Similar shifts were also recorded for Imtrex, which might be due to selection for strains with enhanced fungicide efflux activity. Approximately 10 % of the untreated population showed for epoxiconazole EC_{50} values > 1.0 ppm in 2013; over time this increased to 70 % in 2018, with 15 % of the population showing EC_{50} values > 5.0 ppm. Selection of epoxiconazole insensitivity after fungicide treatment was less pronounced for the populations sampled at Sutton Scotney but the epoxiconazole insensitivity of untreated populations also increased in time (Figure 37).

Figure 36. Epoxiconazole sensitivity profiles of field populations of *Zymoseptoria tritici* sampled from untreated and treated plots at Rosemaund during 2013-2018.
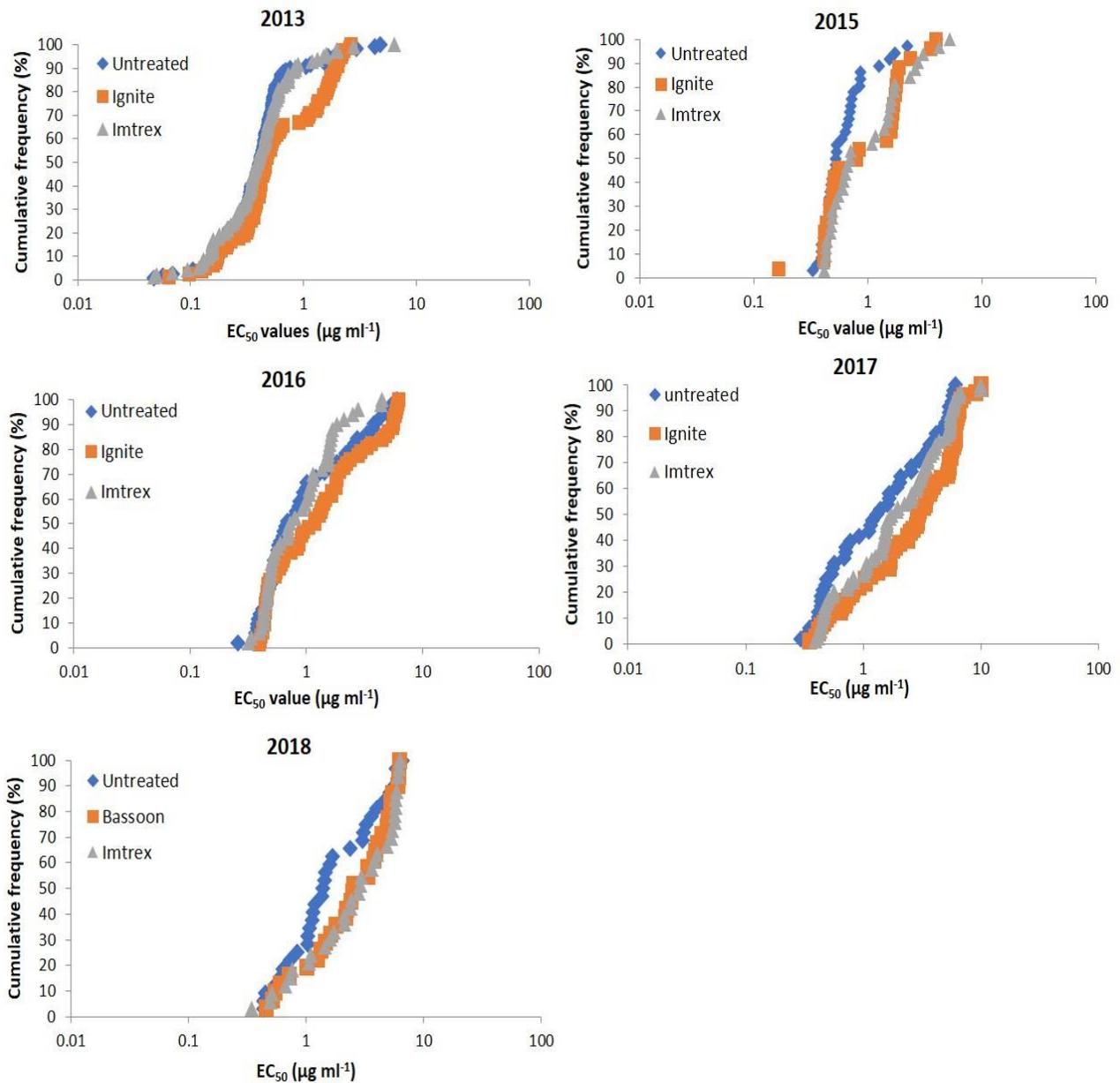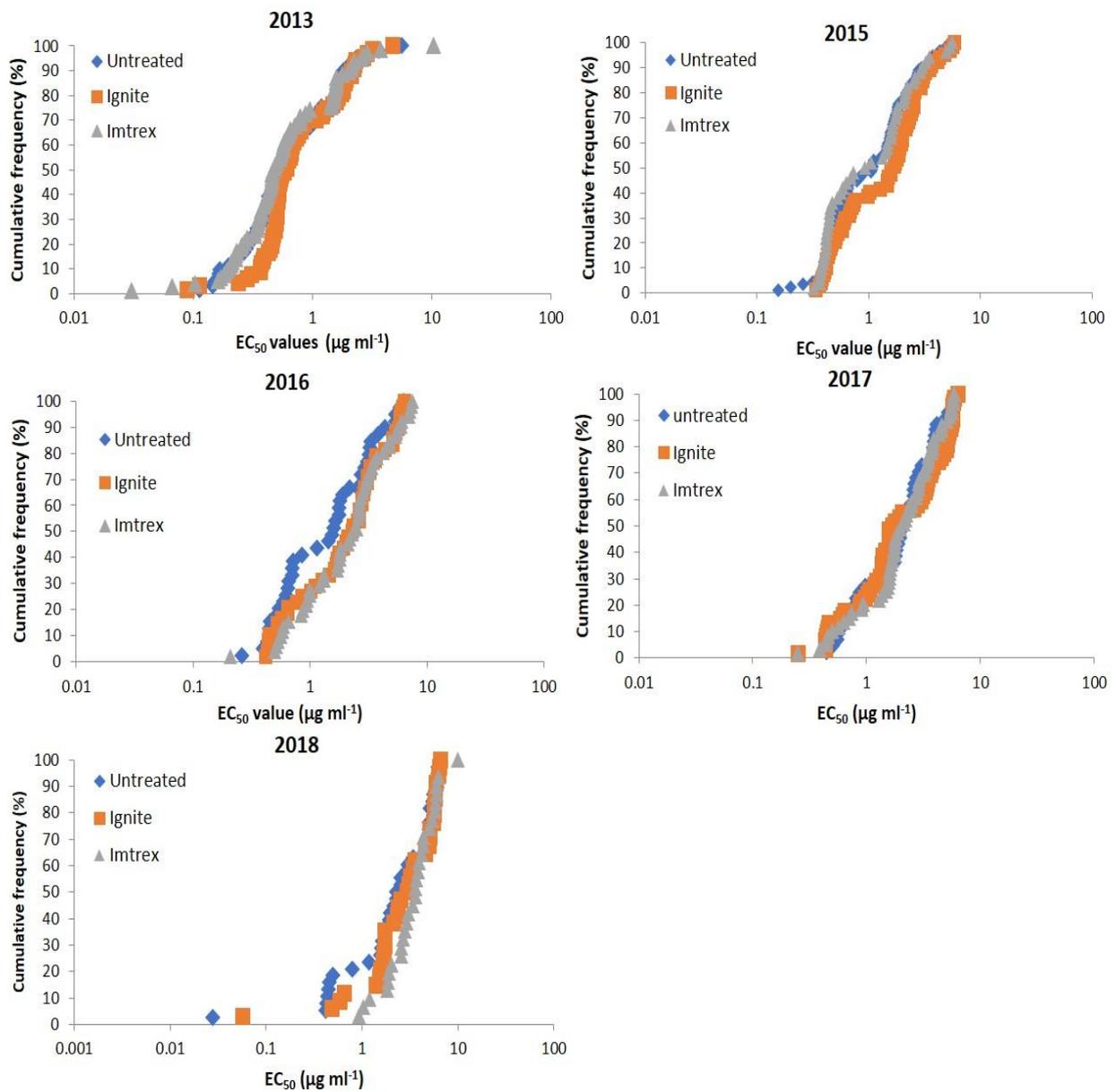
Figure 37. Epoxiconazole sensitivity profiles of field populations of *Zymoseptoria tritici* sampled from untreated and treated plots at Sutton Scotney during 2013-2018.

In comparison with the untreated populations, Imtrex selected strongly for fluxapyroxad insensitivity, with the first shift recorded at Rosemaund in 2015 (Figure 38). Ignite did not select for fluxapyroxad insensitivity in most years with exception of 2016, where, in comparison with Imtrex, a small shift in insensitivity was recorded. The largest fluxapyroxad sensitivity shift was measured in 2017, with 30 % of the untreated population showing bixafen EC_{50} values > 0.3 ppm. The frequency of bixafen insensitive strains (EC_{50} values > 0.3 ppm) was 85 % in the Imtrex treated population. Less than 5 % of the untreated population showed bixafen EC_{50} values > 0.3 ppm in 2013; over time this increased to 65 % in 2018. Highly SDHI insensitive strains (EC_{50} values > 10 ppm) were detected from 2017 onwards at Rosemaund in both treated and untreated plots. Similar trends regarding SDHI insensitivity shifts were measured at Sutton Scotney (Figure 39), where highly SDHI insensitive strains (EC_{50} values > 10 ppm) were detected as early as 2015.

Figure 40 show the results for the shifts in bixafen after treatment of Imtrex at four locations in 2018. The largest shift in SDHI insensitivity was measured in Fife. This population was most sensitive, with 40 % of the untreated population showing bixafen EC_{50} values > 0.3 ppm. The frequency of bixafen insensitive strains was for 90 % in the population treated with Imtrex. highly SDHI insensitive strains (EC_{50} values > 10 ppm) were also detected in the Imtrex treated *Z. tritici* field population of Cardigan (Wales) in 2018, but not in populations sampled in Fife.

Figure 41 show the fungicide sensitivity status of five *Z. tritici* field populations, sampled at Rothamsted, Rosemaund, Sutton Scotney, Carlow (Ireland) and Fife. For epoxiconazole, the Rothamsted and Carlow populations were most insensitive, with the Rosemaund and Fife populations most sensitive. For prothioconazole-desthio, the Sutton Scotney population was most insensitive. For tebuconazole, the Rothamsted population was most insensitive and the Carlow population most sensitive. For prochloraz, the Fife population was least sensitive. This population was also, like in the previous two years (data not shown), least sensitive to fentin chloride, a fungicide and substrate for efflux pumps, with 40 % of strains showing fentin chloride EC_{50} values > 0.2 ppm. Strains with fentin chloride EC_{50} values > 0.2 ppm have increased efflux pump activity, due to presence of different promoter inserts (e.g. 150 and 519 bp) linked with overexpression of MgMFS1 (Omrane *et al.*, 2017). The Carlow population was most insensitive to bixafen with 75 % of the population having EC_{50} values > 0.3 ppm.

Figure 38. Bixafen sensitivity profiling of field populations of *Zymoseptoria tritici* sampled from untreated and treated plots at Rosemaund during 2013-2018.

Figure 39. Bixafen sensitivity profiles of field populations of *Zymoseptoria tritici* sampled from untreated and treated plots at Sutton Scotney during 2013-2018.

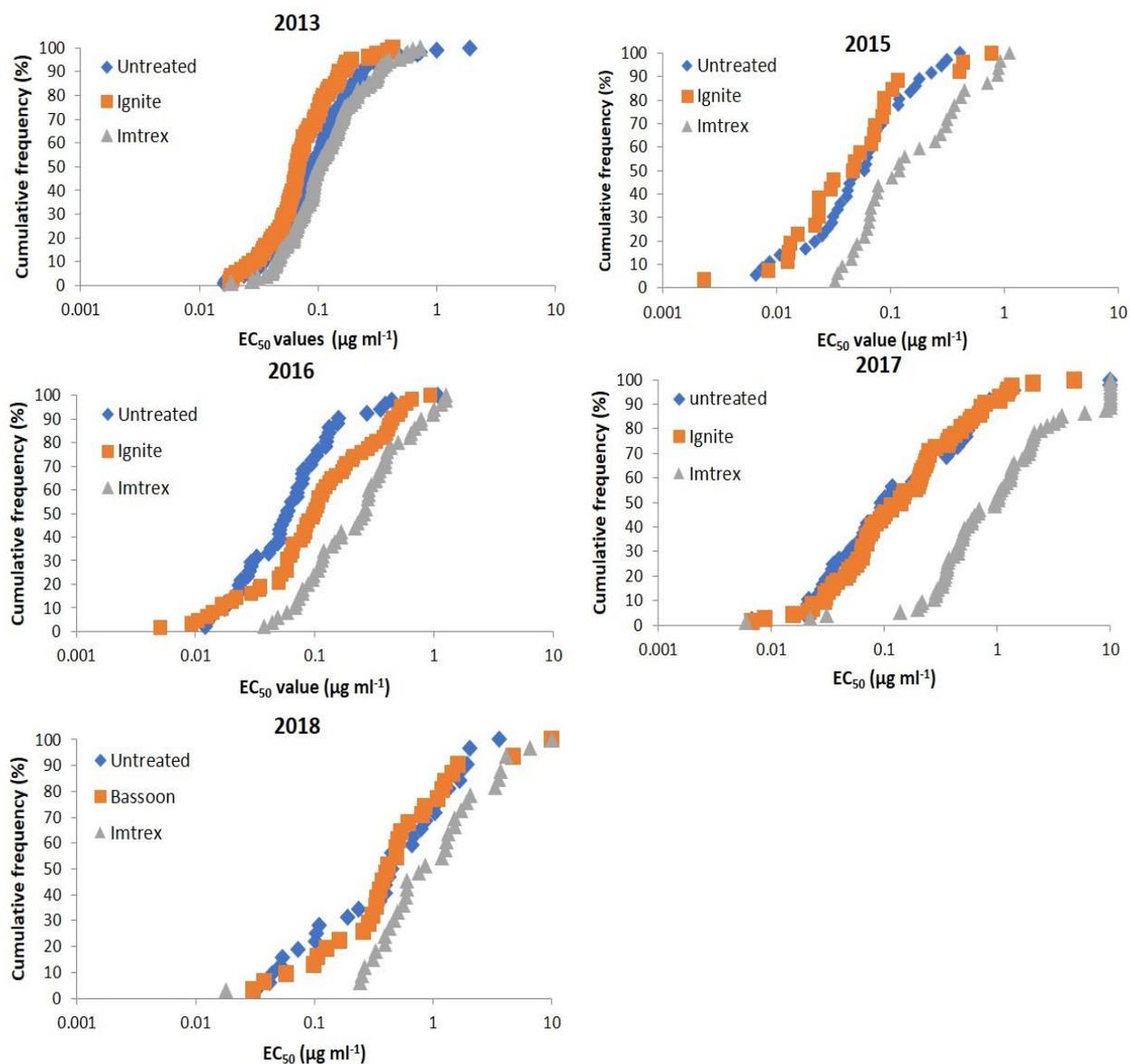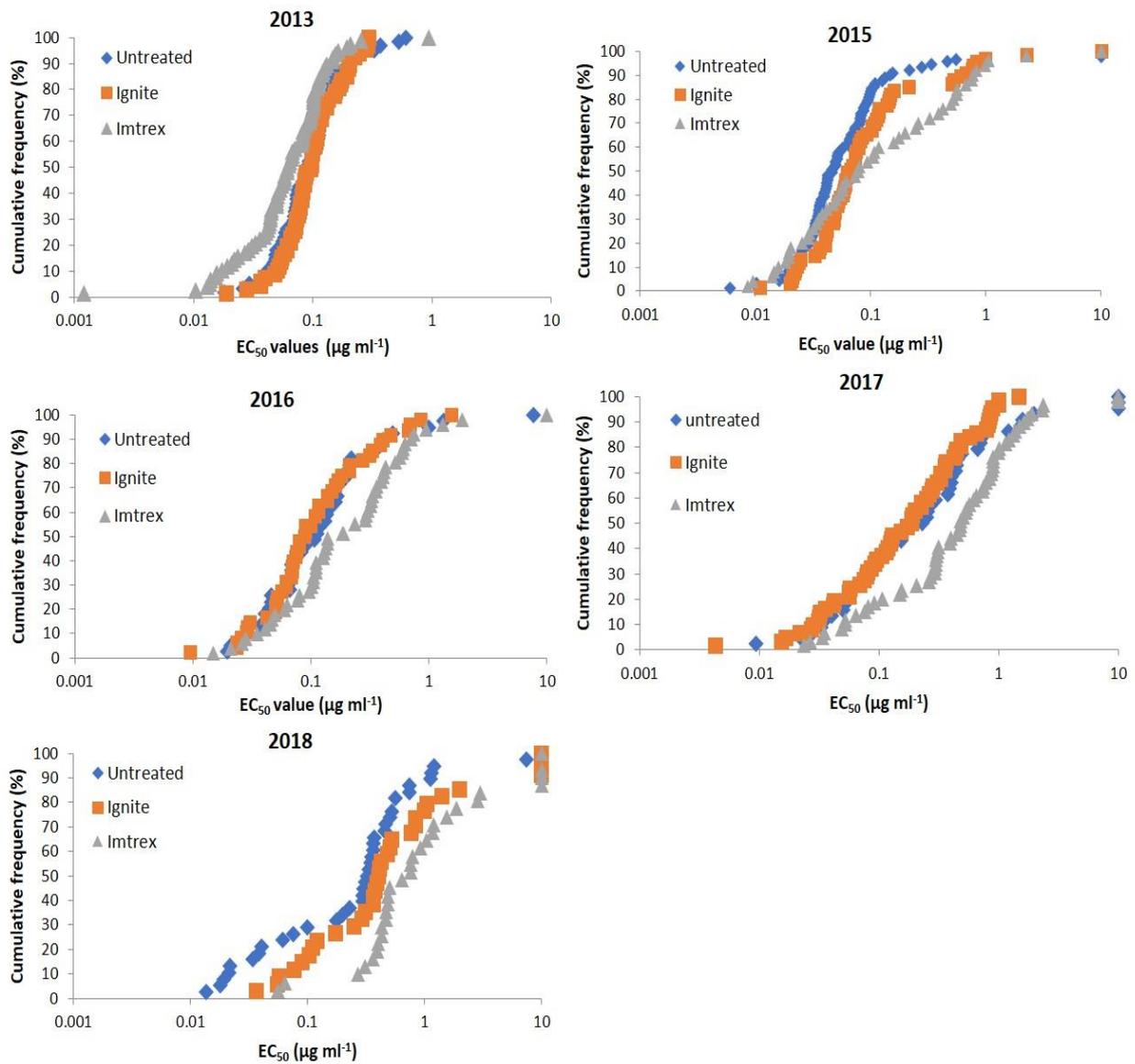**Figure 40.** Bixafen sensitivity profiles of field populations of *Zymoseptoria tritici* sampled from untreated and treated plots at Rosemaund, Cardigan, Sutton Scotney and Fife in 2018.
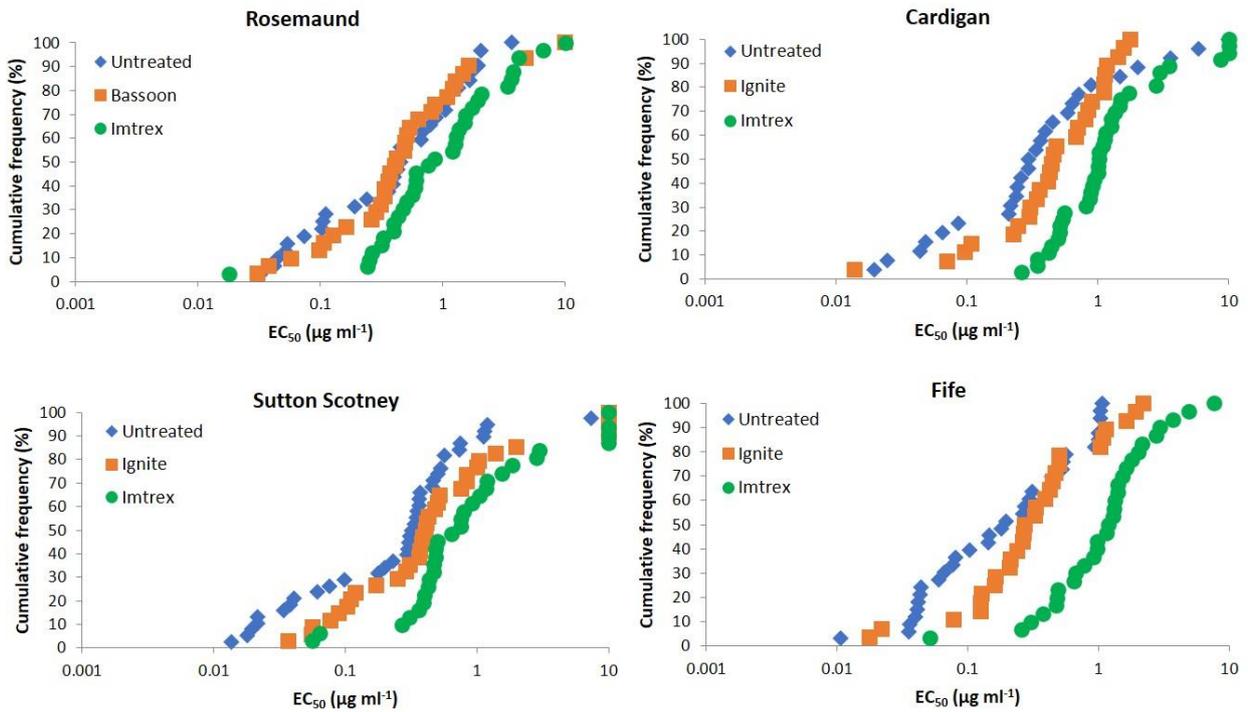
Figure 41. Fungicide sensitivity profiles of field populations of *Zymoseptoria tritici* sampled from untreated fields at Rothamsted, Rosemaund, Sutton Scotney, Carlow (Ireland) and Fife in 2018.
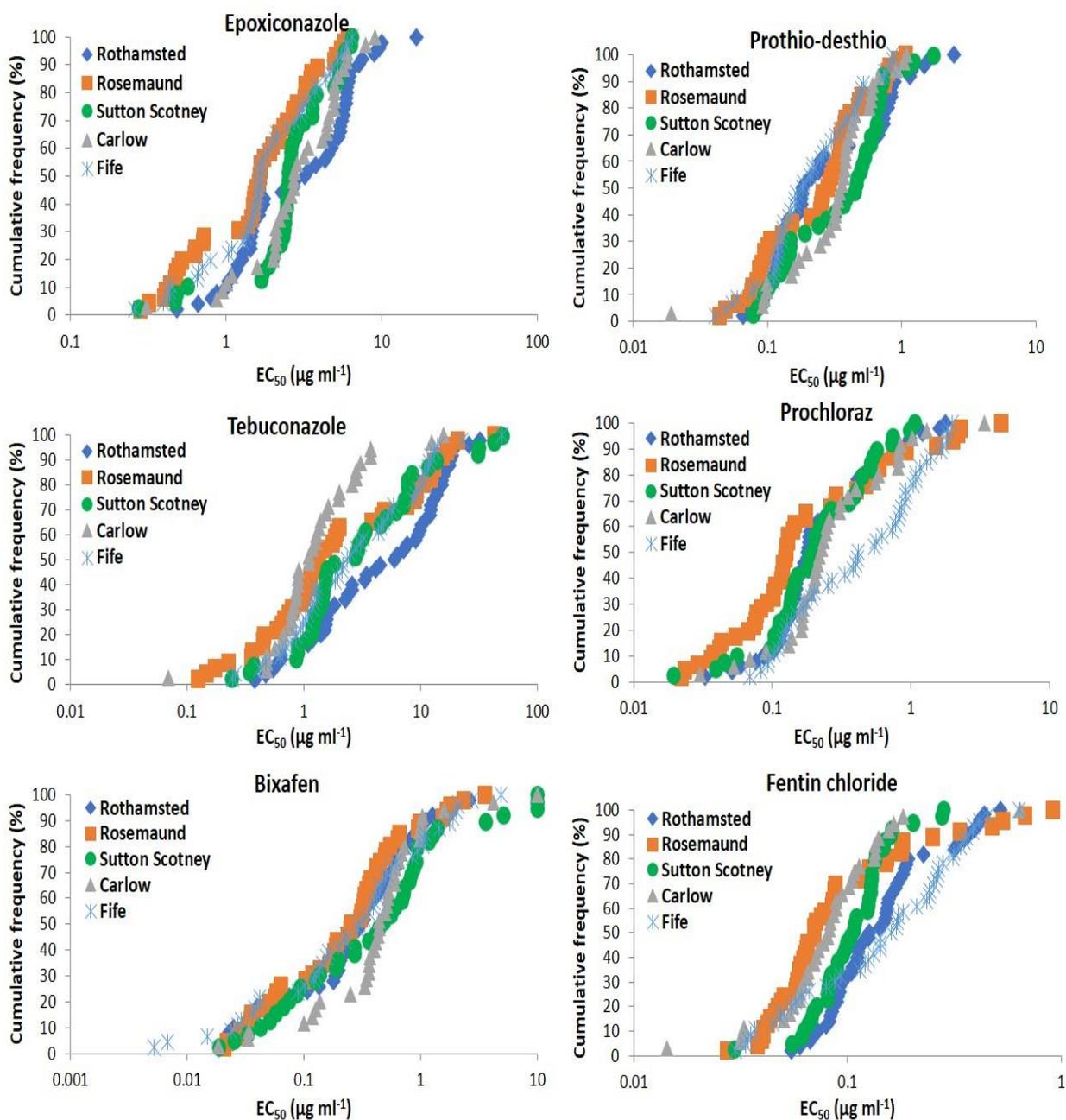
2.4.12.2 Characterisation of azole and SDHI resistance conferring mutations in *Zymoseptoria tritici* field strains sampled from the AHDB Fungicide Performance trials and other field locations during 2013-2018

A selection of *Z. tritici* strains showing EC_{50} values > 0.4 ppm, > 0.8 ppm and > 5.0 ppm for bixafen, prothioconazole-desthio/prochloraz and epoxiconazole, respectively was further characterised by sequencing the azole and SDHI target proteins CYP51 and Sdh subunits B, C and D. Table 13 shows the results for a selection of CYP51 variants that were found. A high number of recent strains with high levels of insensitivity to both epoxiconazole and prothioconazole-desthio, the most important azoles for Septoria control, carry complex CYP51 variants with multiple alterations including S524T. Especially, variants like [L50S, V136A, S188N, A379G, I381V, Δ, S524T], [L50S, V136C, S188N, I381V, Y461H, S524T], [L50S, V136C, S188N, A379G, I381V, Δ, S524T] and [L50S, D134G, V136A, S188N, I381V, Δ, N513K, S524T]. High levels of tebuconazole insensitivity were also found in CYP51-overproducing isolates carrying [L50S, S188N, I381V, Δ, N513K] and, more recently, [L50S, S188N, A379G, I381V, Δ, N513K]. MgMFS1 efflux pump overexpressing strains based on detection of different *MgMFS1* promoter inserts and fentin chloride insensitivity phenotypes can also be found in combination with different CYP51 variants and confer a relatively high level of insensitivity to prochloraz in comparison with other azoles.

Both MgMFS1 efflux pump overexpression and alterations in SdhB, SdhC and/or SdhD were associated with different levels of SDHI insensitivity to bixafen, benzovindiflupyr, fluxapyroxad, isopyrazam and penthiopyrad. Table 14 shows all 18 SDHI insensitive strains (bixafen EC_{50} > 0.3 ppm) with target site alterations that were found in 2015. Most strains (16) were isolated from the AHDB winter wheat fungicide trials at Rosemaund and Sutton Scotney. Two other strains were isolated from samples provided by Velcourt originating from locations near King's Lynn and Warminster. In total, ten different Sdh alterations associated with SDHI insensitivity were identified. Two field strains carried a combination with two key alterations simultaneously, [C-N86K, C-R151T] and [C-N86S, D-D129E]. Strains carrying C-T79N or C-N86S were most frequently detected. Most alterations were found in SdhC with only one SdhB alteration, B-T268I, and one SdhD alteration, D-D129E, found in combination with C-N86S in one strain. Seven different CYP51 variants were detected in the 18 SDHI insensitive strains. These CYP51 variants were linked to high levels of insensitivity to different azoles, showing that they have evolved under selection pressure of both azole and SDHI fungicides applied in mixtures.

Table 13. Selection of CYP51 variants and their average azole sensitivities. Resistance factors of CYP51 variant were calculated as the fold change in mean EC_{50} compared to the mean EC_{50} of four wildtype CYP51 strains carrying no amino acid substitutions.

Isolate - CYP51 variant	Epoxiconazole	Prochloraz	Tebuconazole	Prothio-desthio
Reference strains	EC_{50} values in µg/ml			
Wildtype (no mutations)	0.0029	0.0164	0.072	0.0014
Strains 1994-2008	Resistance factor (RF)			
[Y137F]	2.8	0.9	1.2	0.6
[Y137F & S524T]	12	5.8	4.7	1.8
[L50S, V136A & Y461H]	55	25	4.8	8.8
[L50S, I381V & Y461H]	81	3.5	36	39
[L50S, S188N, I381V, Δ & N513K]	86	3.2	28	15
[L50S, S188N, A379G, I381V, Δ & N513K]	149	1.2	82	21
Strains 2009-2018				
[L50S, S188N, I381V, Δ & N513K] + CYP51↑	389	17	235	92
[L50S, S188N, I381V, Δ & N513K] + CYP51↑ + efflux↑ (MgMFS1)	1428	66	303	207
[L50S, V136A, Y461S & S524T]	206	62	5.4	46
[V136C, I381V, Y461H & S524T]	1111	15	110	93
[L50S, D134G, V136A, Y461S & S524T]	209	12	6.5	112
[L50S, D134G, V136A, I381V & Y461H]	196	11	5.0	102
[L50S, D134G, V136A, I381V & Y461H] + efflux↑ (MgMFS1)	559	62	41	172
[L50S, V136A, I381V, Y461H & S524T]	529	19	20	181
[L50S, V136C, S188N, I381V, Y461H, S524T]	733	7.9	69	85
[L50S, S188N, A379G, I381V, Δ, N513K & S524T]	477	4.5	77	81
[L50S, D134G, V136A, I381V, Y461H & S524T]	809	18	11	336
[L50S, V136A, S188N, A379G, I381V, Δ & S524T]	999	12	21	418
[L50S, V136C, S188N, A379G, I381V, Δ & S524T]	1486	3.3	242	162
L50S, V136A, S188N, A379G, I381V, Δ, N513K & S524T	923	7.7	22	623
[L50S, V136C, S188N, A379G, I381V, Δ, N513K & S524T] + efflux↑	3586	58	318	232
[L50S, D134G, V136A, S188N, A379G, I381V, Δ, N513K & S524T]	1766	11	34	997

Table 14. Sdh variants and their corresponding CYP51 sequences in SDHI insensitive *Zymoseptoria tritici* strains detected late season in 2015.

Strains	Location	Sdh variant[1]	CYP51 variant
ROS42-17	Rosemaund	B-T268I	L50S, S188N, I381V, Δ & N513K↑
317.4	Sutton Scotney	C-T79N	L50S, D134G, V136A, I381V & Y461H
321.2	Sutton Scotney	C-I29V, C-T79N	L50S, S188N, I381V, Δ & N513K↑
101.10	Sutton Scotney	C-I29V, C-T79N, D-R33S	V136C, I381V, Y461H & S524T
CAS36	Sutton Scotney	C-N33T, C-N34T, C-T79N	L50S, S188N, I381V, Δ & N513K↑
V6-9A	King's Lynn	C-N33T, C-N34T, C-T79N, C-V128M	L50S, S188N, I381V, Δ & N513K↑
118.9	Sutton Scotney	C-W80S	L50S, V136C, S188N, I381V, Y461H & S524T
211.8	Sutton Scotney	C-N86K, C-R151T	L50S, V136A, S188N, A379G, I381V, Δ & S524T
CAS27	Sutton Scotney	C-N33T, C-N34T, C-N86S	L50S, V136C, S188N, A379G, I381V, Δ & S524T
104.3	Sutton Scotney	C-N33T, C-N34T, C-N86S	L50S, V136C, S188N, A379G, I381V, Δ & S524T
121.1	Sutton Scotney	C-N33T, C-N34T, C-N86S	L50S, V136C, S188N, A379G, I381V, Δ & S524T
121.3	Sutton Scotney	C-N33T, C-N34T, C-N86S	L50S, V136C, S188N, I381V, Y461H & S524T
317.2	Sutton Scotney	C-N86S, D-D129E	L50S, V136C, S188N, I381V, Y461H & S524T
ROS60-6	Rosemaund	C-R151S	L50S, V136A, S188N, A379G, I381V, Δ & S524T
V9-C23	Warminster	C-N33T, C-N34T, C-I161S	L50S, V136A, S188N, A379G, I381V, Δ & S524T
121.4	Sutton Scotney	C-H152R	L50S, S188N, I381V, Δ & N513K↑
119.11	Sutton Scotney	C-H152R	L50S, D134G, V136A, I381V & Y461H
239.8	Sutton Scotney	C-N33T, C-N34T, C-H152R	L50S, D134G, V136A & I381V

[1] Alterations linked with SDHI resistance are marked in red.

In vitro SDHI cross-resistance testing showed that strains carrying C-H152R and the double alteration [C-N86S, D-D129E] had the highest levels of insensitivity to bixafen, boscalid, fluxapyroxad, penthiopyrad and Solatenol (benzovindiflupyr)) (Table 15). Results for fluopyram were less clear with, for example, wildtype strain R15-46 also showing a high EC_{50} value of 7.105 ppm. As expected, the lowest EC_{50} values were recorded for wild-type Sdh variant without overexpression of MgMFS1.

Eleven out of the 18 strains carrying Sdh alterations and an additional C-H152R strain provided by DuPont were also tested in the glasshouse for *in planta* fungicide sensitivity to Imtrex (Figure 42). A good correlation was found with the *in vitro* assays. Three out of four C-H152R strains and Sdh variant [C-N86S, D-D129E] could not be controlled using a full dose of Imtrex. Other variants were well controlled at rates lower than a quarter rate. Both wildtype Sdh variants with overexpression of MgMFS1 were controlled at the 1/32 rate, whereas most three out of four wildtype Sdh variants without MgMFS1 overexpression were controlled at the 1/128 rate.

Table 15. *In vitro* SDHI sensitivity testing of *Zymoseptoria tritici* field strains carrying different Sdh variants or overexpressing MgMFS1. Sensitivities are displayed as EC_{50} values in µg ml^{-1}. Technical grade products with exception of Solatenol (formulated benzovindiflupyr) were used.

Strain	Sdh alteration[1]	Bixafen	Boscalid	Fluopyram	Fluxapyroxad	Penthiopyrad	Solatenol
IPO323	wt	0.003	0.079	0.042	0.021	0.001	0.008
LN591	wt	0.011	0.080	0.057	0.021	0.046	0.006
R15-46	wt	0.042	0.372	7.105	0.069	0.030	0.009
V212-2	wt	0.050	0.182	0.114	0.048	0.013	0.018
121.3	C-N86S	0.196	0.283	0.071	0.497	0.571	0.083
UPL1B-41	wt, MgMFS1	0.214	1.106	3.906	0.278	0.346	0.069
ROS42-17	B-T268I	0.313	0.871	0.823	1.023	0.909	0.162
121.1	C-N86S	0.315	0.512	0.888	1.065	1.249	0.139
CAS27	C-N86S	0.315	0.659	1.07	1.080	1.600	0.196
118.9	C-W80S	0.319	1.306	0.799	0.546	0.864	0.062
CAS36	C-T79N	0.357	1.202	3.053	0.982	0.835	0.130
V9C-23 A	C-I161S	0.364	0.695	2.657	0.472	0.899	0.130
V6-9A	C-T79N	0.370	1.752	0.976	1.028	0.757	0.150
321.2	C-T79N	0.371	1.091	1.110	1.051	0.810	0.127
ROS60-6	C-R151S	0.405	0.562	0.487	1.187	1.524	0.125
211.8	C-N86K & C-R151T	0.462	0.761	0.690	1.184	1.926	0.103
NT321.17	wt, MgMFS1	0.603	1.376	1.715	0.624	0.692	0.061
101.10	C-T79N	0.657	1.240	1.593	1.795	1.777	0.125
317.2	C-N86S, D-D129E	1.277	>20	2.038	5.124	6.158	0.397
119.11	C-H152R	>10	>20	2.534	>20	>20	0.457
121.4	C-H152R	>10	4.203	3.827	11.0	>20	0.557
239.8	C-H152R	>10	10.0	8.487	12.0	>20	0.632

[1]Only key alterations linked to SDHI insensitivity are shown

The number and frequencies of different Sdh variants have increased over time with 28 different Sdh variants being recorded in the UK so far (Table 16). Only strains with bixafen EC_{50} values > 2.0 ppm are being further checked for presence of new mutations. C-H152R strains are often detected after treatment but monitoring has shown that the frequencies have continued to be low at the start of the season. Studies by Gutièrrez-Alonso *et al*. (2017) have shown that a fitness cost is associated with this mutation. Isolates carrying multiple Sdh alterations are still rare in populations despite a high level of insensitivity recorded for Sdh variant [C-N86S, D-D129E]. Higher than expected bixafen EC_{50} values were found for strains with combinations of Sdh variants and MgMFS1 overexpression in 2018. Two new Sdh variants with high resistance factors to all SDHI fungicides and previously reported in lab UV mutants, B-H267L and C-N86K (Fraaije *et al*., 2012; Scalliet *et al*., 2012), were found in field strains during late season in 2018. Further monitoring is required to see if these Sdh variants are further spreading and accumulating in field populations.

Figure 42. *In planta* SDHI sensitivity testing of *Zymoseptoria tritici* field strains carrying different Sdh variants. One day preventative application of Imtrex, four leaves per strains with symptoms recorded 21 days after inoculation. Red: all 3 leaves show large areas of necrotic tissues with abundant pycnidia; orange: only 1 or two leaves with some pycnidia-bearing necrotic areas; yellow; mainly areas with discoloration due to Septoria infection.
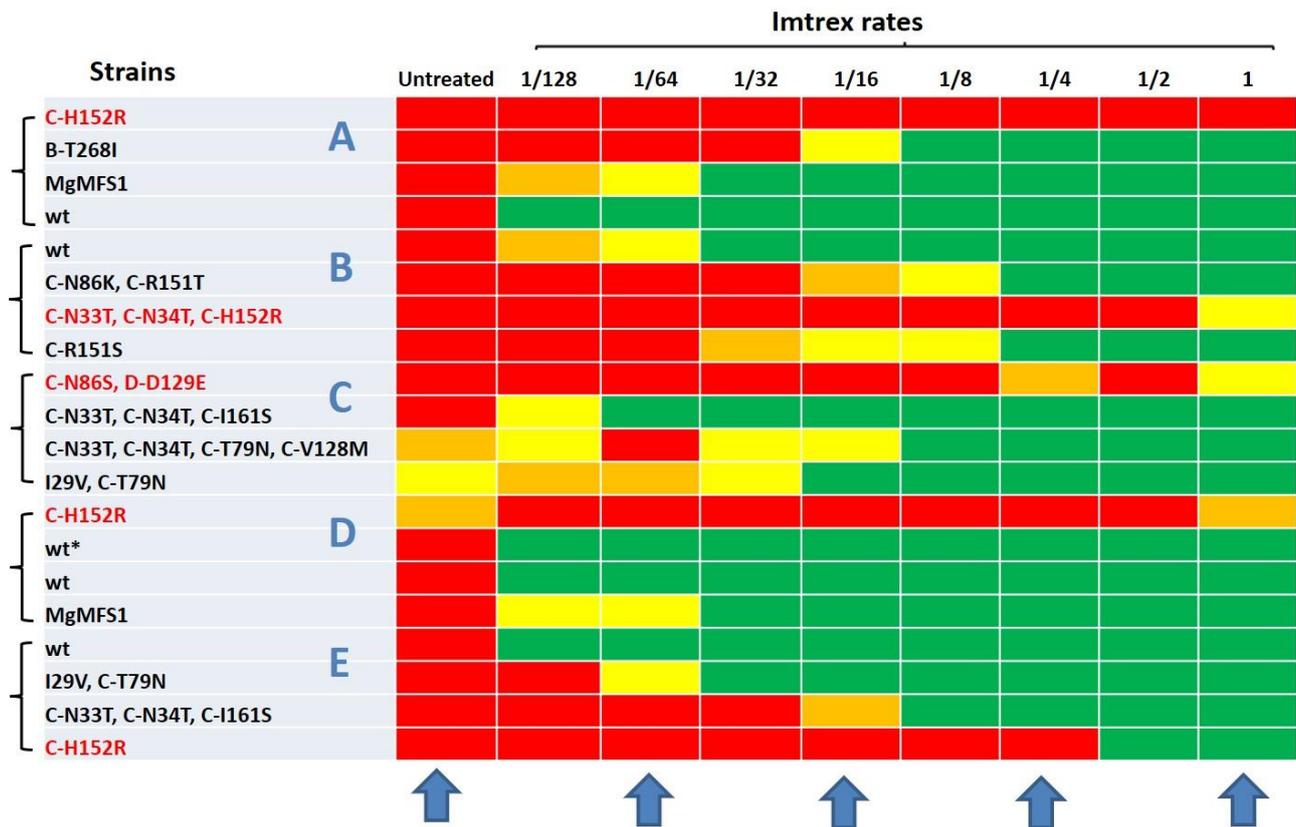
Tray E
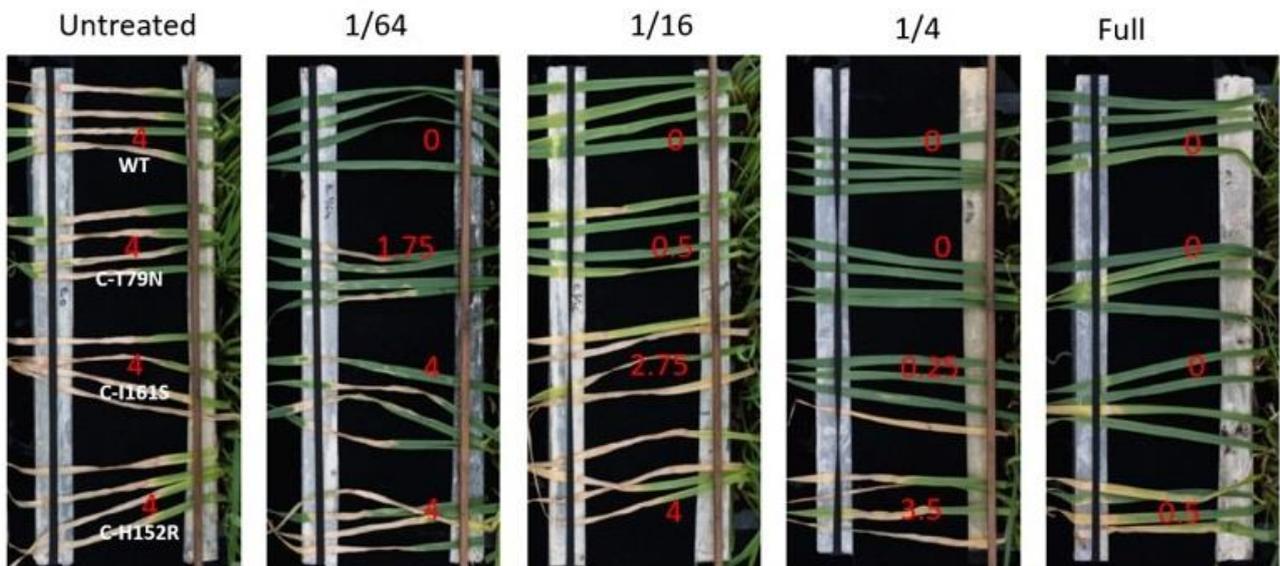
Table 16. Detection of Succinate dehydrogenase (SdhB, C and D) alterations linked with SDHI insensitivity in *Zymoseptoria tritici* UK field isolates.

Key Sdh mutation	Strains (n)	First detection	Location
B-H267L*	1	2018	Hampshire
B-H267R	1	2018	Hampshire
B-T268I	2	2015	Herefordshire, Hertfordshire
B-I269V	2	2018	Hertfordshire
B-N271T	1	2016	Dyfed
C-T79F	1	2018	Hertfordshire
C-T79I	4	2016	Hampshire, Fife, Hertfordshire
C-T79N	41	2015	Dyfed, Hampshire, Herefordshire, Hertfordshire, Kent, Lincolnshire, Norfolk
C-W80S	14	2015	Hampshire, Fife, Hertfordshire, Herefordshire
C-N86K*	2	2018	Hampshire
C-N86K + C-R151T	1	2015	Hampshire
C-N86S	57	2015	Dorset, Fife, Hampshire, Herefordshire, Suffolk, Hertfordshire
C-N86S + D-D129E*	1	2015	Hampshire
C-N86T	1	2019	Hertfordshire
C-N86T + D-D129E	1	2017	Herefordshire
C-V88M	1	2018	Hertfordshire
C-R151G	2	2017	Fife
C-R151M	2	2016	Fife, Hertfordshire
C-R151S	6	2015	Herefordshire, Kent, Hertfordshire
C-R151T	5	2015	Hampshire, Kent, Hertfordshire
C-H152R*	46	2015	Devon, Hampshire, Kent, Fife, Dyfed, Hertfordshire, Herefordshire
C-I161S	1	2015	Wiltshire
C-V166M	1	2019	Hertfordshire
C-G170E	1	2017	Kent
D-I50F	1	2018	Hertfordshire
D-I50N	1	2018	Fife
D-D129E	3	2015	Dorset, Gloucestershire, Fife
D-D129G	1	2017	Fife

*Highly SDHI insensitive Sdh variants in red

2.4.13 Rothamsted early season fungicide sensitivity monitoring of *Zymoseptoria tritici* isolates

Fifty leaves with Septoria symptoms, each leaf sampled 3 metres apart, were each year collected from untreated winter wheat fields at Rothamsted Farm at the start of the season (January-February).

2.4.13.1 Azole and SDHI sensitivity shifts in *Zymoseptoria tritici* field populations sampled at Rothamsted during 2012-2019

Each year 40-50 strains were isolated from 50 leaves sampled from untreated fields at Rothamsted during 2012 to 2019. The sensitivity of each strain to a selection of azole fungicides (epoxiconazole, prochloraz, prothioconazole-desthio and tebuconazole) and a SDHI fungicide (bixafen) was determined (Figure 43). Clear shifts in both azole and SDHI sensitivity was observed during this period.

For tebuconazole, a bimodal distribution of EC_{50} values was observed. Up to 2016, 40 to 60 % of the population showed EC_{50} values < 1.0 ppm and 30 to 50 % of the population had EC_{50} values > 3.0 ppm. Since 2017, only up to 20 % of the population showed EC_{50} values < 1.0 ppm, with > 40 % of the isolates showing EC_{50} values > 10 ppm. A bimodal trend was also observed for the prochloraz. Highly sensitive strains with EC_{50} values < 0.01 ppm were detected till 2016. More than 10 % of the population show EC_{50} values > 0.4 ppm since 2018. Regarding the prothioconazole-desthio and epoxiconazole sensitivity, the populations tested showed a similar trend with more pronounced shifts during 2014-2016 and 2017-2018. For epoxiconazole, most strains showed EC_{50} values < 1.0 ppm till 2016. A large fraction of the population (> 20%) had EC_{50} values > 5.0 ppm after 2018. For prothioconazole-desthio, most strains produced EC_{50} values < 0.1 ppm till 2016. A large fraction of the population (> 30%) showed EC_{50} values > 0.3 ppm after 2018.

No trend in bixafen sensitivity was measured when comparing the results for the populations tested during 2012-2016 with most strains recording EC_{50} values < 0.3 ppm. The first shift towards insensitivity was observed in 2017, with 20 % of the population having an EC_{50} value > 0.3 ppm. Further shifting occurred in 2018 and 2019 with a larger proportion of the population recording EC_{50} value > 0.3 ppm; (50 and 70 % for 2018 and of 2019, respectively) and, for the first time, some strains showing EC_{50} values > 1.0 ppm.

Figure 43. Azole and SDHI fungicide sensitivity profiles of field populations of *Zymoseptoria tritici* sampled at Rothamsted during 2012 (n=46), 2013 (n=59), 2014 (n=41), 2015 (n=46), 2016 (n=49), 2017 (n=41), 2018 (n=50) and 2019 (n=49)
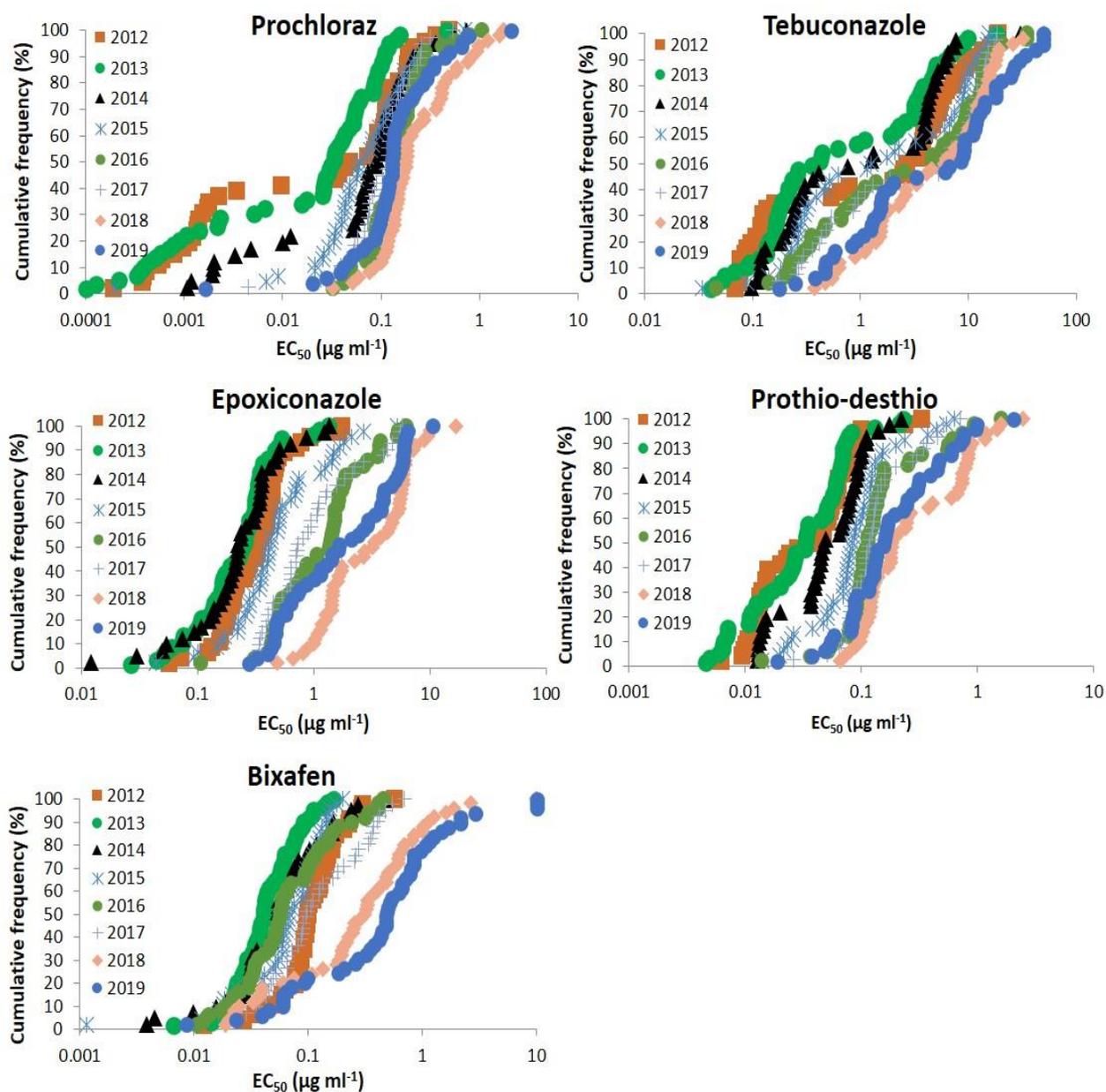
2.4.13.2 Characterisation of azole resistance conferring mutations in *Zymoseptoria tritici* field strains sampled at Rothamsted during 2012-2019

The azole fungicide sensitivity shifts detected in the Rothamsted field populations (Figure 43) were linked to the evolution and accumulation of particular CYP51 variants in these populations as shown in Table 18. In total, 37 different CYP51 were detected over the ten-year period. The dynamics of the ten most frequently occurring CYP51 variants are displayed in Figure 44.

Table 18. Distribution of CYP51 variants within untreated *Zymoseptoria tritici* populations sampled at Rothamsted at the start of the season during 2010-2019

CYP51 variants[1]	2010 (n=40)	2011 (n=41)	2012 (n=45)	2013 (n=38)	2014 (n=40)	2015 (n=38)	2016 (n=34)	2017 (n=35)	2018 (n=44)	2019 (n=47)
L50S-V136A-Y461H	0	0	1	1	0	0	0	0	0	0
L50S-V136A-Y461S	0	1	0	0	0	1	0	0	0	0
L50S-I381V-Y459D	8	0	0	0	0	0	0	0	0	0
L50S-I381V-Y459S	1	0	0	0	0	0	0	0	0	0
L50S-I381V-Y461H	8	8	3	0	0	0	0	0	0	0
D107V-I381V-Y461H	1	0	0	0	0	0	0	0	0	0
D107V-I381V-N513K-S524T	0	1	0	0	1	0	0	0	0	0
L50S-S188N-I381V-Y461H	0	1	0	0	0	0	0	0	0	0
L50S-S188N-Δ-N513K	1	0	0	0	0	0	0	0	0	0
D134G-V136A-S188N-Δ	0	1	0	0	0	0	0	0	0	0
V136C-I381V-Y461H-S524T	0	0	0	0	0	1	0	0	3	1
V136C-I381V-Δ-S524T	0	0	0	0	0	0	0	0	0	1
L50S-D134G-V136G-Y461S	0	0	0	0	0	1	0	0	0	0
L50S-V136A-S188N-Δ-N513K	1	0	0	0	0	0	0	0	0	0
L50S-S188N-I381V-Δ-N513K	1	0	0	0	0	0	0	0	0	0
L50S-V136C-S188N-Δ-N513K	0	1	0	0	0	0	0	0	0	0
L50S-D134G-V136A-I381V-Y459S	0	0	0	0	0	0	0	0	1	0
L50S-D134G-V136A-I381V-Y461H	1	5	12	19	15	12	7	8	2	10
L50S-V136A-S188N-Δ-S524T	0	0	3	1	3	1	0	0	0	0
L50S-V136A-I381V-Y461H-S524T	0	0	1	2	2	2	1	0	1	0
L50S-V136C-I381V-Y461H-S524T	0	0	0	0	0	0	1	0	0	0
V136C-S188N-I381V-Δ-S524T	0	0	0	0	0	0	0	0	1	0
L50S-V136A-S188N-I381V-Δ-N513K	1	0	0	0	0	0	0	0	0	0
L50S-D134G-V136A-I381V-Y461H-S524T	0	1	0	0	0	2	0	2	5	7
L50S-S188N-A379G-I381V-Δ-N513K	17	22	19	10	7	1	1	1	0	1
L50S-I381V-S188N-DEL-N513K-CYP↑	0	0	5	4	11	13	15	14	14	10
L50S-S188N-A379G-I381V-Δ-S524T	0	0	0	0	0	0	0	1	0	0
L50S-S188N-A379G-I381V-Y459D-S524T	0	0	0	0	0	0	0	0	0	1
L50S-V136C-S188N-I381V-Y461H-S524T	0	0	0	0	1	1	0	5	2	4
L50S-S188N-I381V-Δ-N513K-S524T	0	0	0	0	0	1	0	0	0	0
L50S-S188N-A379G-I381V-Δ-N513K-S524T	0	0	0	1	0	1	0	0	0	0
L50S-S188N-A379G-I381V-Y459D-N513K-S524T	0	0	0	0	0	0	0	0	0	1
L50S-V136C-S188N-A379G-I381V-Δ-S524T	0	0	0	0	0	1	0	2	4	8
L50S-V136C-S188N-A379G-I381V-Y461H-S524T	0	0	0	0	0	0	5	0	0	0
L50S-V136A-S188N-A379G-I381V-Δ-S524T	0	0	0	0	0	0	3	1	5	2
L50S-V136A-S188N-A379G-I381V-Δ-N513K-S524T	0	0	1	0	0	0	0	0	2	1
L50S-D134G-V136A-A379G-I381V-Δ-N513K-S524T	0	0	0	0	0	0	1	1	4	0

↑, CYP51 overexpressing isolates; Δ, 6 bp deletion resulting in deletion of two amino acids of the CYP51 protein (Y459 & G460)

Figure 44. Dynamics of frequently occurring CYP51 variants (≥ 5 % in at least one year) in untreated Septoria field populations at Rothamsted during 2010-2019
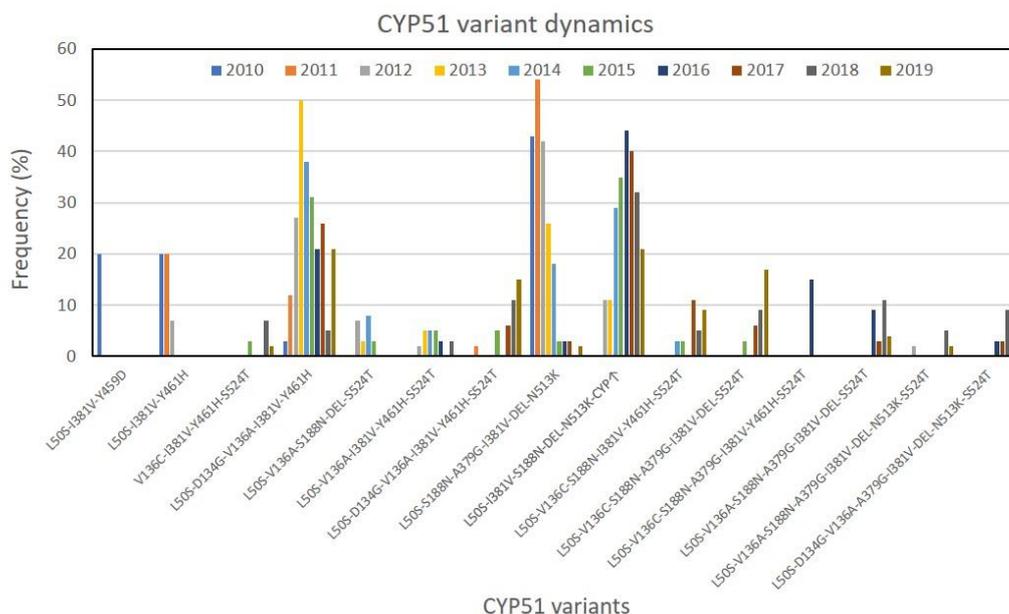
The CYP51 variant dynamics showed that CYP51 variants get replaced over time. Variant [L50S, I381V, Y461H] with only three alterations got replaced by variant [L50S, S188N, A379G, I381V, Δ, N513K]. This variant was followed up by [L50S, D134G, V136A, I381V, Y461H] and then by the CYP51 over-expressing variant [L50S, S188N, I381V, Δ, N513K]. Finally, increasing amounts of variants [L50S, V136C, S188N, A379G, I381V, Δ, S524T] and [L50S, D134G, V136A, I381V, Y461H, S524T] have been detected since 2017 and these variants might, depending on the azole selection pressure and their sensitivity levels (see Table 19), become most common in the population soon. Tebuconazole, epoxiconazole and prothioconazole are currently the most used azoles to control fungal diseases on wheat in the UK.

Table 19. Average azole resistance factors of the most commonly occurring CYP51 variants in Rothamsted field populations of *Zymoseptoria tritici* during 2010-2018.

CYP51 variant	Resistance Factor (RF)[1]			
	Epoxiconazole	Prochloraz	Tebuconazole	Prothio-desthio
[L50S, I381V, Y461H]	81	3.5	36	39
[L50S, S188N, A379G, I381V, Δ, N513K]	149	1.2	82	21
[L50S, D134G, V136A, I381V, Y461H]	196	11	5.0	102
[L50S, S188N, I381V, Δ, N513K] + CYP51↑	389	17	235	92
[L50S, D134G, V136A, I381V, Y461H, S524T]	809	18	11	336
[L50S, V136C, S188N, A379G, I381V, Δ, S524T]	1486	3.3	242	162

[1]Resistance factors of strains belonging to different CYP51 variants were calculated as the fold change in mean EC_{50} compared to the mean EC_{50} of four wildtype CYP51 strains carrying no amino acid substitutions. Mean EC_{50} values of the wild-type CYP51 variants were 0.0029, 0.0164, 0.0720 and 0.0014 ppm for epoxiconazole, prochloraz, tebuconazole and prothioconazole-desthio, respectively.

2.4.13.3 Characterisation of SDHI resistance conferring mutations in *Zymoseptoria tritici* field strains sampled at Rothamsted during 2012-2019

The SDHI fungicide sensitivity shifts detected in the Rothamsted field populations (Figure 43) since 2017 were linked to evolution and accumulation of strains with overexpression of MgMFS1 and/or presence of mutations in SdhB, C and/or D. Sdh variants were found in two strains of the 2017 Rothamsted population (n = 41), C-T79N and C-R151S. In 2018 (n = 50), 19 out of 21 isolates with bixafen EC_{50} > 0.4 ppm carried Sdh alterations (B-I269V (1), C-T79F (1), C-T79I (1), C-T79N (4), C-W80S (2), C-N86S (7), C-V88M (1), C-R151S (1) and D-I50F). In 2019 (n = 49), 30 out of 31 isolates with bixafen EC_{50} > 0.4 ppm carried Sdh alterations (B-I269V (1), C-T79N (5), C-W80S (3), C-N86S (11), C-N86T (1), C-R151S (2), C-R151T (2), C-R151M (1), C-H152R (3) and C-V166M (1).

2.4.14 DNA-based monitoring of *Sdh* mutations in UK and Irish field populations of *Zymoseptoria tritici*

SNP detection Pyrosequencing assays were developed to detect and quantify Sdh mutations in field populations of *Z. tritici*. Due to its detection threshold of 3-5 %, genotyping assays targeting C-T79N, C-W80S, C-N86S and C-H152R, the most frequently occurring mutations in 2015, were prioritised. Results for early season monitoring from 2016 to 2019 are shown in Figure 45. *Sdh* mutations were not detected in 2016. From 2017 onwards, the frequencies for C-T79N and C-N86S have been increasing, with a more sporadic detection of C-W80S and absence of C-H152R at the start of the season at a detection threshold of 5 %. Frequencies of 19.2 and 24.3 % were measured for C-T79N and C-N86S, respectively, in the 2019 Rothamsted population. This is close to the frequencies of 10.2 and 22.4 % for C-T79N and C-N86S, respectively, determined using with *Sdh* sequencing of individual strains (n = 49). The overall frequency of *Sdh* mutations in the Rothamsted population was 61.2 % due to the contribution of eight other mutations at low frequencies (below 5 %).

Figure 45. Dynamics of the most frequently occurring *Sdh* mutations in Septoria field populations sampled from untreated fields at the start of the season during 2016-2019. Circles represent single fields.
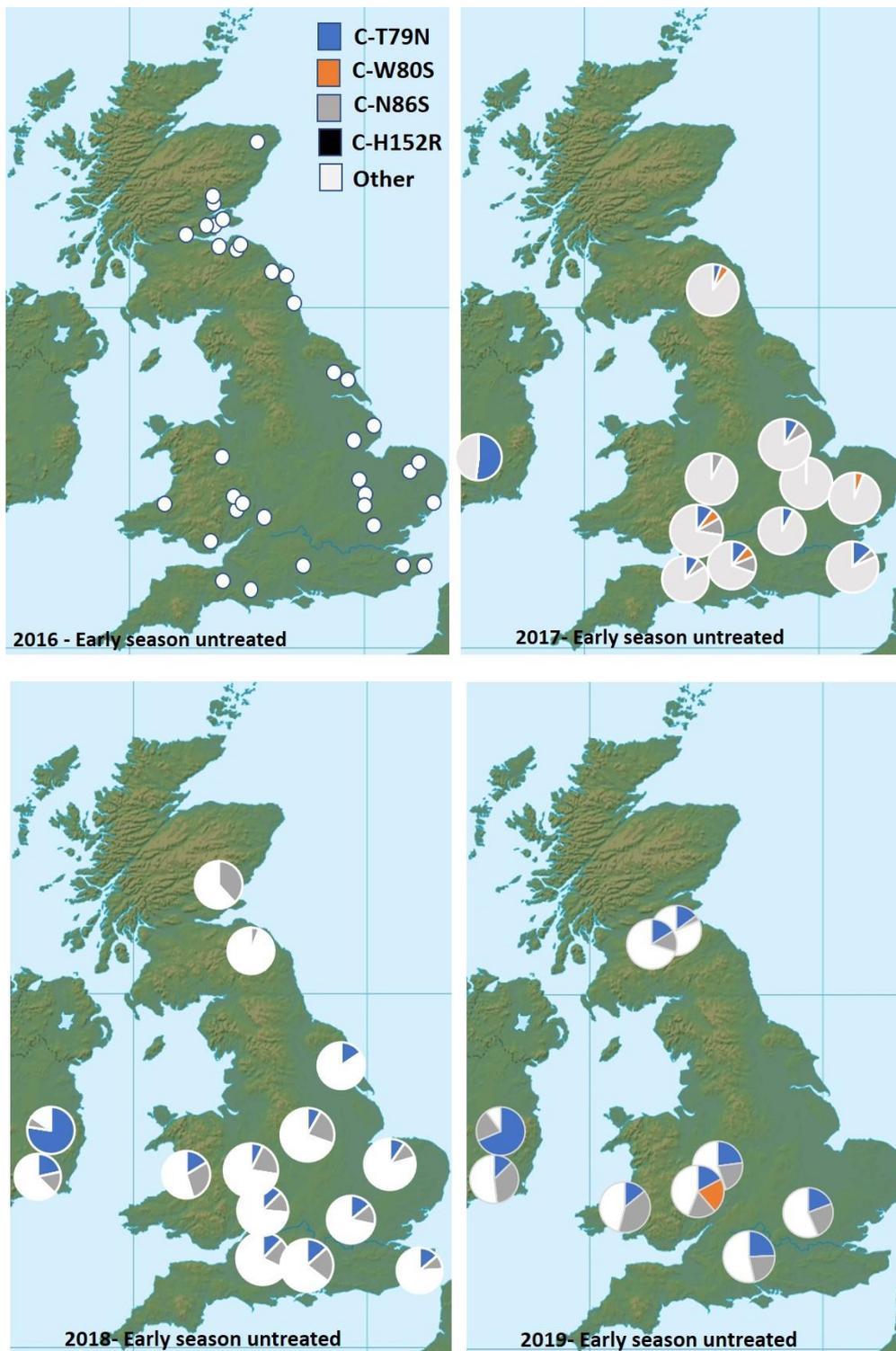
2.4.15 Research by industry partners

All Industry partners (BASF, Bayer CropScience, DuPont and Syngenta) regularly presented field trial data on cereal pathogens and results from their annual fungicide sensitivity monitoring programs in Europe during the lifespan of the project. The monitoring results and use recommendations are also archived and updated every year on the Fungicide Resistance Action Committee (FRAC) websites through the working groups for sterol biosynthesis inhibitors (SBIs) (https://www.frac.info/working-group/sbi-fungicides/general-use-recommendations) and SDHIs (https://www.frac.info/working-group/sdhi-fungicides/general-use-recommendations). Cereal pathogens included in these monitoring programs are Septoria tritici blotch (*Z. tritici*), powdery mildew (*Blumeria graminis* spp.)*,* brown rust (*Puccinia triticina*), eyespot (*Oculimacula* spp.), tan spot (*Pyrenophora tritici-repentis*), Rhynchosporium scald (*Rhynchosporium secalis*) and net blotch (*Pyrenophora teres*). Regarding azole and SDHI sensitivity shifts, several important papers have recently been published by Industry partners. Huf *et al*. (2018) proposed a new nomenclature for *CYP51* haplotypes in *Z. tritici*. They found 33 different *CYP51* haplotypes in a collection of 331 field isolates sampled across Europe in 2016. Nine haplotypes represented 85% of all isolates and showed a heterogeneous distribution across Europe. CYP51 variants with S524T were associated with a decreased sensitivity to all azoles and were found at frequencies of around 5%. In addition to the Sdh alterations found in our studies (see Table 9), Scalliet *et al*. (2012) reported several new alterations in UV lab mutants of *Z. tritici* using selected after exposure to different SDHIs. These were B-S218F, B-P220L/T, B-N225I/H, B-H267Q, B-N271K, C-T79I, C-S83G, C-A84I/V, C-R87C, C-V88D, C-H145R, C-H152R and D-D129S. Rehfus *et al*. (2017) reported on the presence of *Sdh* mutations in European field isolates of *Z. tritici* and its impact on the sensitivity to various succinate dehydrogenase inhibitors. The following Sdh alterations were found: C-T79N (2012), C-W80S (2012), C-N86S (2013), B-N225T (2014), B-T268I (2015), C-V166M (2015) and C-H152R (2015). Rehfus *et al*. (2019) monitored DMI and SDHI fungicide sensitivity shifts and the underlying resistance mechanisms in European field populations of *Ramularia collo-cygni*. Sdh alterations B-H266Y/R, B-T267I, B-I268V, C-N87S, C-H146R and C-H153R were found with increasing frequencies in populations since 2014. In addition, 15 different CYP51 haplotypes were detected in DMI-adapted isolates of *R. collo-cygni* sampled from 2009 to 2017. SDHI resistant Sdh variants have also been reported for *P. teres* since 2012. Rehfus *et al*. (2016) reported the detection B-H277Y (2012), C-

N75S (2013), C-G79R (2013), C-H134R (2013), C-S135R (2013), D-D124E/N (2013), D-H134R (2013), D-D145G (2013) and D-E178E (2014).

2.5 DISCUSSION

The evolution of resistance to azole and SDHI fungicides in field populations of *Z. tritici* has been a gradual, on-going process and dependent on the selection pressure imposed by azoles, SDHI and multi-sites used to control Septoria tritici blotch. There has been a stepwise erosion of efficacy of DMI (azole) fungicides since the mid-1990s, accompanied by the emergence and accumulation of genetic changes in the sterol 14α-demethylase (CYP51) target protein. Analysis of archived Broadbalk samples revealed the first azole-resistance conferring mutation leading to CYP51 amino acid substitution Y137F emerged as early as in 1991 (Fraaije *et al*., 2011). This mutation was replaced by alterations at codons 459-461 of the CYP51 protein during the mid and late 1990s. More complex CYP51 variants harbouring alterations at codons 459-461 in combination with key alterations such as V136A (2000), A379G (2003), I381V (2001) and/or, most recently, S524T (2008) have evolved since 2000. Mutations can affect the direct binding of azoles differentially and/or restore CYP51 enzyme activity (Mullins et al., 2011). Although many different CYP51 variants have been detected, a selection of these have accumulated at high frequencies in UK populations due to selection pressure of different azoles over time (Table 19). Our detailed studies on *Z. tritici* populations sampled at Rothamsted showed the following succession steps of frequently occurring CYP51 variants: [L50S, I381V, Y461H] > [L50S, S188N, A379G, I381V, Δ, N513K] > [L50S, D134G, V136A, I381V, Y461H] > CYP51 over-expressing variant [L50S, S188N, I381V, Δ, N513K] > [L50S, V136C, S188N, A379G, I381V, Δ, S524T] and [L50S, D134G, V136A, I381V, Y461H, S524T]. A similar evolution is currently also ongoing in Ramularia populations with combinations of CYP51 mutations such as Y403C/Y405H (equivalent to positions 459 and 461 of *Z. tritici* CYP51 (see Mair *et al*., 2016)), I325T and I328L (position 381 in ZtCYP51) playing an important role (Rehfus *et al*., 2019).

The practical importance of *in vitro* fungicide sensitivity monitoring in this study and Fraaije *et al*. (2006) was demonstrated by Blake *et al*. (2017). New resistant strains are often detected by resistance monitoring and laboratory phenotyped/genotyped before changes in field performance are detected. Strong relationships between laboratory tests and field performance (in field dose-response curves for DMI and QoI fungicides) were demonstrated and could aid translation between laboratory and field for other fungicide groups.

In vitro experimental evolution lab experiments on resistance to SDHI fungicides in *Z. tritici* were like MBC fungicides highly predictive for what might evolve in field populations (Hawkins & Fraaije, 2016). Sdh mutations resulting in B-H267L, B-I269V, C-N86K/S, C-T79I, C-H152R, D-D129E/G (Fraaije *et al.* 2012; Scalliet *et al*., 2012) have all been found in the field (Table 16), with new mutations still evolving. Of these Sdh variants, C-H152R field strains are highly insensitive to all SDHI fungicides tested (Dooley et al., 2016; Rehfus *et al*., 2017). Interestingly, Gutièrez-Alonso *et al*. (2017) also showed that *Z. tritici* lab mutants with C-H152R are likely to carry a fitness cost due to their inability to compete for growth with other lab mutant Sdh variants in the absence of SDHI fungicides or in the presence of SDHI fungicides at low doses. Fluopyram binds differently to the Sdh binding pocket formed by SdhB C and D (Fraaije et al., 2012) and different *Sdh* mutations conferring resistance have been found in lab studies (Scalliet et al., 2012). Yamashita & Fraaije (2018) reported that a non-target site resistance mechanism operating within mitochondria can confer insensitivity to the SDHIs fluopyram and isofetamid. The underlying resistance mechanism was recently elucidated by Steinhauer *et al*. (2019). A sub population of *Z. tritici* carry a second, dispensable SdhC paralog with an unique Qp-site residue that affects binding of a subclass of SDHI fungicides. The expression of this SdhC paralog was variable in field strains depending on the presence of promoter transposon insertions.

This project had two strategies for fungicide resistance management: 1, Use azoles to protect SDHIs from resistance, but minimise selection of azole resistant strains; 2, Use SDHIs to protect azoles from resistance, but minimise selection of SDHI resistant strains. In the absence of SDHI resistance during 2010-2014, field trials were used to investigate the first strategy; the effect of an SDHI reducing selection for DMI insensitive strains. Modelling and experimental evidence shows that if resistance is evolving against one fungicide with a single mode of action, mixing with another fungicide with a different single mode of action should reduce selection for insensitive pathogen strains but strains with insensitivity against both are likely to evolve (Hobbelen et al., 2013; Van den Bosch *et al*., 2014). In further studies as part of the monitoring project we have seen that SDHI insensitive Sdh variants also simultaneously carry the latest evolved azole insensitive CYP51 variants (Table 14). Results from the field trials show that reduction of the number of azole treatments per crop has the greatest effect on azole resistance build-up. A solo treatment of full label dose azole gave the largest increase in frequency of azole insensitive strains as selection is dose dependent. Increasing doses of SDHI (isopyrazam in this study) as a

mixture partner resulted in decreasing selection for azole insensitive strains (as measured by selection of prochloraz for D134G and/or V136A alleles and against A379G and/or I381V alleles and *vice versa* for tebuconazole selection). Using SDHIs as mixing partner will reduce the selection for insensitivity of the azole fungicides. The larger the SDHI dose in the mixture, the smaller the rate of selection for azole insensitivity. A reduction of the azole dose in the mixture helps reduce selection. For example, the additional benefit of reducing the prochloraz dose for alleviating selection for the V136A mutation was quite small. This follows from the generally weak effect of prochloraz dose on selection seen experimentally. If the opposite is valid for using azoles to protect SDHIs in mixtures then we can expect that mixtures of SDHIs with robust doses of azoles will help increase the effective life of the SDHI fungicides with little deleterious effect on the effective life of the azole component in the mixture (Van den Bosch et al., 2015).

2.5.1 Future resesearch

Monitoring the SDHI and azole sensitivity profiles of field populations must continue as the evolution of CYP51 and Sdh variants in *Z. tritici* field populations in response to selection by azole and SDHI fungicide is an on-going process. This information can be used directly to advise farmers on the most efficient use of azoles and SDHIs in balanced mixture-based spray programmes. With new SDHIs (e.g. isoflucypram (Iblon) and pydiflumetofen (Adepidyn)), a new mode of action for cereals, the QiI fenpicoxamid (Inatreq) (Owen *et al*., 2017), and metyltetraprole (Pavecto), a QoI fungicide able to control cytochrome *b* G143A strains of *Z. tritici* (Suemoto *et al*., 2019), and a new azole, mefentrifluconazole (Revysol) (Tesha *et al*. (2019), coming into the market it is important to establish in which direction the future evolution of Sdh and CYP51 goes and if multiple azoles/SDHIs and/or other (new) MoA in balanced mixtures can alleviate the selection pressure. With chlorothalonil being withdrawn from the market, it is becoming even more important to test anti-resistance strategies based on existing and new MOA (single and multi-sites) as well as incorporating host resistance. The tools developed during this project can be applied directly in follow-up studies. Further advances can be made in the detection of novel fungicide resistant alleles using PCR amplicon next generation sequencing (NGS) techniques and quantification using NGS and digital PCR allowing detection at much lower detection thresholds. This would enable implementation of anti-resistance strategies and/or measures before resistance is further spread and established in the field.

2.6 ACKNOWLEDGEMENTS

2.7 REFERENCES

- Avenot, H.F. and Michailides, T.J. (2010) Progress in understanding molecular mechanisms and evolution of resistance to succinate inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Prot.* 29, 643-651.

- Avenot, H., Sellam, A. and Michailides, T. (2009) Chararacterization of mutations in the membrane-anchored subunits AaSDHC and AaSDHD of succinate dehydrogenase from *Alternaria alternata* isolates conferring resistance to the fungicide boscalid. *Plant Pathol.* 58, 1134-1143.

- Bearchell SJ, Fraaije BA, Shaw MW & Fitt BDL (2005) Wheat archive links long-term fungal population dynamics to air pollution. *Proceedings of the National Academy of Sciences of the United States of America* (PNAS) 102, 5438-5442.

- Bertelsen JR, De Neergaard E & Smeedegaard-Petersen (2001) Fungicidal effects of azoxystrobin and epoxiconazole on phyllosphere fungi, senescence and yield of winter wheat. *Plant Pathology* 50: 190-205.

- Blake J, Gosling P, Fraaije BA, Burnett FJ, Knight SM, Kildea S & Paveley ND (2017) Changes in field dose-response curves for DMI and QoI fungicides against *Zymoseptoria tritici*, related to laboratory sensitivity phenotyping and genotyping assays. *Pest Management Science* 74:302–313.

- Bounds P, Blake J, Paine F, Knight S, Burnett F, Kildea S & Gosling P (2016). Fungicide performance on winter wheat. *AHDB Project Report* No 550, 42 pp.

- Clark, W. S. (2006) *Septoria tritici* and azole performance. In *Fungicide resistance: are we winning the battle but losing the war? Aspects Appl. Biol.* 78, 127-132.

- Cools HJ, Bayon C, Atkins S, Lucas JA & Fraaije BA (2012) Over-expression of the sterol 14α-demethylase gene (MgCYP51) in *Mycosphaerella graminicola* isolates

confers a novel azole fungicide sensitivity phenotype. *Pest Management Science* 68, 1034-1040.

- Cools HJ & Fraaije BA (2008). Mechanisms of resistance to azole fungicides in *Mycosphaerella graminicola*. *Pest Management Science* 64, 681-684

- Cools, H. J. and Fraaije, B. A. (2013) Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest Management Science* 69: 150–155

- Cools HJ, Bayon C, Atkins S, Lucas JA & Fraaije BA (2012) Over-expression of the sterol 14α-demethylase gene *(MgCYP51)* in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Management Science* 68, 1034-1040

- Dooley H, Shaw MW, Mehenni-Ciz J, Spink J, Kildea S, (2016) Detection of *Zymoseptoria tritici* SDHI-insensitive field isolates carrying the SdhC-H152R and SdhD-R47W substitutions. *Pest Management Science* **72**:2203–2207.

- Fraaije BA, Burnett FH, Clark WS and Lucas JA (2006) Development and field testing of fungicide anti-resistance strategies, with particular reference to strobilurin QoI group of fungicides. *HGCA Project report* No 392, 112 pp.

- Fraaije, B.A., Lucas, J.A., Clark, W.S., and Burnett, F.J. (2003) QoI resistance development in populations of cereal pathogens in the UK. In *Proceedings of the BCPC Congress, Crop Science and Technology 2005*, Vol. 2, 689-694. Alton, UK: BCPC.

- Fraaije BA, Burnett FJ, Clark WS, Motteram J & Lucas JA (2005a). Resistance development to Qo Inhibitors in populations of *Mycosphaerella graminicola* In: Modern fungicides and antifungal compounds IV, eds Dehne HW, Gisi U, Kuck K-H, Russell P and Lyr H, Chapter 8, 63-71. BCPC: Alton, UK.

- Fraaije BA, Burnett FJ, Cools HJ, Clark WS, Paveley N, Norman K and Lucas JA (2011) Understanding evolution and selection of azole resistance mechanisms in UK populations of *Mycosphaerella graminicola*. *HGCA project report* No. 475, 124 pp.

- Fraaije, B.A., Cools, H.J., Fountaine, J., Lovell, D.J., Motteram, J., West, J.S. and Lucas, J.A. (2005b) The role of ascospores in further spread of QoI-resistant cytochrome *b* alleles (G143A) in field populations of *Mycosphaerella graminicola*. *Phytopathology,* 95, 933-941.

- Fraaije, B.A., Cools, H.J., Kim, S-H, Motteram, J, Clark, W.S. and Lucas, J.A. (2007) A novel substitution I381V in the sterol 14α-demethylase (CYP51) of

Mycosphaerella graminicola is differentially selected by azole fungicides. *Mol. Plant Pathology* 8, 245-254

- Gisi, U., Pavic, L., Stanger, C., Hugelshofer, U. and Sierotski, H. (2005). Dynamics of *Mycosphaerella graminicola* populations in response to selection by different fungicides. In Modern Fungicides and Antifungal Compounds IV (Dehne, H.W., Gisi, U., Kuck, K-H., Russell, P. E. and Lyr, H. eds), pp. 89-101. Alton, UK: BCPC.

- Griffin, M. J. and Fischer, N. (1985) Laboratory studies on benzimidazole resistance in *Septoria tritici*. EPPO *Bulletin* 15, 505–511.

- Gutiérrez-Alonso, O, Hawkins, NJ, Cools, HJ, Shaw, MW and Fraaije, BA (2017) Dose-dependent selection drives lineage replacement during experimental evolution of SDHI fungicide resistance in *Zymoseptoria tritici*. *Evolutionary Applications* 10, 1055-1066

- Hawkins NJ & Fraaije BA (2016) Predicting resistance by mutagenesis: lessons from 45 years of MBC resistance. *Frontiers in Microbiology* 7: 1814.

- Hobbelen PHF, Paveley ND, Oliver RP and van den Bosch F (2013) The usefulness of fungicide mixtures and alternation for delaying the selection for resistance in populations of *Mycosphaerella graminicola* on winter wheat: a modeling analysis. *Phytopathology* 103: 690-707.

- Hollomon D, Cooke L & Locke T. (2002) Maintaining the effectiveness of DMI fungicides in cereal disease control strategies. HGCA report No. 275, 24 pp.

- Huang, L-S., Sun, G., Cobessi, D., Wang, A.C., Shen, J.T., Tung, E.Y., Anderson, V.E. and Berry, E.A. (2006) 3-Nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. *J. Biol. Chem.* 281, 5965-5972.

- Huf A, Rehfus A, Lorenz KH, Bryson R, Voegele RT and Stammler (2018) Proposal for a new nomenclature for *CYP51* haplotypes in *Zymoseptoria tritici* and analysis of their distribution in Europe. *Plant Pathology* 67, 1706-1712

- Keon, J., Antionow, J., Carzaaniga, R., Deller, S., Ward, J., Baker, J. M., Beale, M. H., Hammond-Kosack, K., Rudd, J. J. (2007) Transcriptional adaptation of *Mycosphaerella graminicola* to programmed cell death (PCD) of its susceptible wheat host. *Mol Plant-Microbe Interact* 20, 179-193.

- Leroux & Walker Leroux, P. and Walker, A-S. (2011). Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Manag. Sci.* 67, 44-59.

- Lucas JA & Fraaije BA (2008). The final straw: crop archives and the recent history of cereal pathosystems. In: *BSPP Presidential Meeting proceedings, cereal pathosystems* (pp. 103-115), UK: University of London. British Society of Plant Pathology/Home Grown Cereals Authority.

- Lucas JA, Hawkins NJ & Fraaije BA (2015) The evolution of fungicide resistance. *Advances in Applied Microbiology* 90, 29-92.

- Mair W, Lopez-Ruiz F, Stammler G, Clark W, Burnett F, Hollomon D, Ishii H, Thind TS, Brown JKM, Fraaije BA, Cools H, Shaw M, Fillinger S, Terrado EM, Schnabel G, Mehl A & Oliver RP (2016) Proposal for a unified nomenclature for target site mutations associated with resistance to fungicides. *Pest Management Science* 8, 1449-59

- Matsson, M., Ackrell, B.A., Cochran, B. and Hederstedt, L. (1998) Carboxin resistance in Paracoccus denitrificans conferred by a mutation in the membrane-anchor domain of succinate:quinine reductase. *Arch. Microbiol.* 170, 27-37.

- Miyamoto, T., Ishii, H., Stammler, G., Koch, A., Ogawara, T., Tomita, Y., Fountaine, J.M., Ushio, S., Seko, T. and Kobori, S. (2010) Distribution and molecular characterization of *Corynespora cassiicola* isolates resistant to boscalid. *Plant Pathol*. 59, 873-881.

- Mullins JGL, Parker JE, Cools HJ, Martel CM, Togawa RC, Lucas JA, Fraaije BA, Kelly DE, Kelly SL, (2011) Molecular Modelling of the Emergence of Azole Resistance in *Mycosphaerella graminicola*. *PLoS One* **6**: 1-11 (e20973).

- Omrane S, Sghyer H, Audeon C, Lanen C, Duplaix C, Walker AS and Fillinger S (2015) Fungicide efflux and the MgMFS1 transporter contribute to the MDR phenotype in *Zymoseptoria tritici* field isolates. *Environmental Microbiology* 17, 2805-2823.

- Omrane S, Audéon C, Ignace A, Duplaix C, Aouini L, Kema G, Walker AS, Fillinger S, 2017. Plasticity of the MFS1 promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*. *Appl. & Env Science* 5:1-18.

- Owen JW, Yao C, Myung K, Kemmitt G, Leader A, Meyer KG, Bowling AJ, Slanec T, Kramer VJ (2017) Biological characterization of fenpicoxamid, a new fungicide with utility in cereals and other crops. *Pest Management Science* 73: 2005-2016.

- Parker JE, Warrilow AGS, Cools HJ, Martel CM, Nes WD, Fraaije BA, Lucas JA, Kelly DE & Kelly SL (2011). Prothioconazole binds to *Mycosphaerella graminicola* CYP51 by a different mechanism compared to other azole antifungals. *Applied and Environmental Microbiology* 77, 1460-1465.

- Pijls, C. F. N., Shaw, M. W., and Parker, A. (1994) A rapid test to evaluate in vitro sensitivity of *Septoria tritici* to flutriafol, using a microtitre plate reader. Plant Pathol. 43, 726-732.

- Rehfus A, Matusinsky P, Strobel D, Bryson R and Stammler G (2019) Mutations in target genes of succinate dehydrogenase inhibitors and demethylation inhibitors in *Ramularia collo-cygni* in Europe, *Journal of Plant Diseases and Protection*, 10.1007/s41348-019-00246-4.

- Rehfus A, Miessner S, Achenbach J, Strobel D, Bryson R and Stammler G (2016) Emergence of SDHI resistance of *Pyrenophora teres* in Europe. *Pest Management Science* 72: 1977-1988.

- Rehfus A, Strobel D, Bryson R, Stammler G, 2017. Mutations in *sdh* genes in field isolates of *Zymoseptoria tritici* and impact on the sensitivity to various succinate dehydrogenase inhibitors. *Plant Pathology* 67: 175-180.

- Scalliet G, Bowler J, Luksch T, Kirchhofer-Allan L, Steinhauer D, Ward K, Niklaus M, Verras A, Csukai M, Daina A and Fonné-Pfister R (2012) Mutagenesis and functional studies with succinate dehydrogenase inhibitors in the wheat pathogen *Mycosphaerella graminicola*. *PLoS One* 7(4): e35429.

- Suemoto H, Matsuzaki Y and Iwahashi F (2019) Metyltetraprole, a novel putative complex III inhibitor, targets known QoI-resistant strains of *Zymoseptoria tritici* and *Pyrenophora teres. Pest Management Science* on-line (DOI 10.1002/ps.5288)

- Sierotzki H and Scalliet G (2013) A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. Phytopathology 103: 880-887

- Skinner, W., Bailey, A., Renwick, A., Keon, J., Gurr, S. and Hargreaves, J. (1998) A single amino-acid substitution in the iron–sulphur protein subunit of succinate dehydrogenase determines resistance to carboxin in *Mycosphaerella graminicola* (*Septoria tritici*). *Current Genetics* 34, 393–398.

- Stammler, G., Carstensen, M., Koch, A., Semar, M., Strobel, D. and Schlehuber, S. (2008). Frequency of different CYP51-haplotypes of *Mycosphaerella graminicola* and their impact on epoxiconazole-sensitivity and -field efficacy. Crop Prot 27:1448-1456.

- Steinhauer D, Salat M, Frey R, Mosbach A, Luksch T, Balmer D, Hansen R, Widdison S, Logan G, Dietrich RA, Kema GHJ, Bieri S, Sierotzki H, Torriani SFF, Scalliet G (2019) A dispensable paralog of succinate dehydrogenase subunit C mediates standing resistance towards a subclass of SDHI fungicides in *Zymoseptoria tritici*. *bioRxiv* http://dx.doi.org/10.1101/616904

- Stergiopoulos, I., Van Nistelrooy, J. G. M., Kema, G. H. J. and De Waard, M. A. (2003) Multiple mechanisms account for variation in base-line sensitivity to azole fungicides in field isolates of *Mycosphaerella graminicola*. *Pest Manag. Sci.* 59, 1333-1343.

- Shima, Y., Ito, Y., Kaneko, S., Hatabayaashi, H., Watanabe, Y., Adachi, Y., and Yabe, K. (2009) Identification of three mutant loci conferring carboxin-resistance and development of a novel transformation system in *Aspergillus oryzae*. *Fungal Genet. Biol.* 46, 67-76.

- Stergiopoulos, I., Van Nistelrooy, J. G. M., Kema, G. H. J. and De Waard, M. A. (2003) Multiple mechanisms account for variation in base-line sensitivity to azole fungicides in field isolates of *Mycosphaerella graminicola*. *Pest Manag. Sci.* 59, 1333-1343.

- Tesha SA, Tesha JM, Fegert I, Buesen R, Schneider S, Mentzel T, van Ravenzwaay B, Stinchcombe S (2019) Innovative selection approach for a new antifungal agent mefentrifluconazole (Revysol®) and the impact upon its toxicity profile. *Regulatory Toxicology and Pharmacology* 106: 152-168

- Van den Bosch F, Oliver R, Van den Berg F, Paveley N (2014) Governing principles can guide fungicide-resistance management tactics. *Annual Review of Phytopathology* 52: 175-195

- Van Den Bosch, F., Fraaije, B. A., Oliver, R., Van Den Berg, F. and Paveley, N. 2015. The use of mathematical models to guide fungicide resistance management decisions. in: Hollomon, D. W. and Ishii, H. (ed.) *Fungicide resistance in plant pathogens* Springer, Tokyo. pp. 49-62.

- Yamashita M and Fraaije BA (2018) Non-target site SDHI resistance is present as standing genetic variation in field populations of *Zymoseptoria tritici*. *Pest Management Science* 74: 672-681

PROJECT OUTPUT

Published papers

- Blake J, Gosling P, Fraaije BA, Burnett FJ, Knight SM, Kildea S & Paveley ND, (2017) Changes in field dose-response curves for DMI and QoI fungicides against *Zymoseptoria tritici*, related to laboratory sensitivity phenotyping and genotyping assays. *Pest Management Science* 74:302–313.

- Cools HJ, Bayon C, Atkins S, Lucas JA & Fraaije BA (2012) Over-expression of the sterol 14α-demethylase gene *(MgCYP51)* in *Mycosphaerella graminicola* isolates

confers a novel azole fungicide sensitivity phenotype. *Pest Management Science* 68, 1034-1040.

- Cools HJ & Fraaije BA (2013) Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest Management Science* 69, 150-155.

- Cools HJ, Hawkins NJ & Fraaije BA (2013). Constraints on the evolution of azole resistance in plant pathogenic fungi. *Plant Pathology* 62, 36-42.

- Cools HJ, Mullins JGL, Fraaije BA, Parker JE, Kelly DE, Lucas JA & Kelly SL (2011). Impact of recently emerged sterol 14α-demethylase (CYP51) variants of *Mycosphaerella graminicola* on azole fungicide sensitivity. *Applied and Environmental Microbiol*ogy 77, 3830-3837.

- Fraaije BA, Bayon C, Atkins S, Cools HJ, Lucas JA & Fraaije MW (2012). Risk assessment studies on Succinate Dehydrogenase Inhibitors, the new weapons in the battle to control Septoria leaf blotch in wheat. *Molecular Plant Pathology* 13, 263-275.

- Gutiérrez-Alonso, O, Hawkins, NJ, Cools, HJ, Shaw, MW and Fraaije, BA (2017) Dose-dependent selection drives lineage replacement during experimental evolution of SDHI fungicide resistance in *Zymoseptoria tritici*. *Evolutionary Applications* 10, 1055-1066

- Hawkins NJ & Fraaije BA (2016) Predicting resistance by mutagenesis: lessons from 45 years of MBC resistance. *Frontiers in Microbiology* 7: 1814.

- Hawkins NJ and Fraaije BA (2018) Fitness penalties in the evolution of fungicide resistance. *Annual Review of Phytopathology* 56: 339-360.

- Hobbelen PHF, Paveley ND, Fraaije BA, Lucas JA & van den Bosch F. (2011) Derivation and validation of a model to predict selection for fungicide resistance. *Plant Pathology* 60, 304-313.

- Lucas JA, Hawkins NJ & Fraaije BA (2015) The evolution of fungicide resistance. *Advances in Applied Microbiology* 90, 29-92.

- Mair W, Lopez-Ruiz F, Stammler G, Clark W, Burnett F, Hollomon D, Ishii H, Thind TS, Brown JKM, Fraaije BA, Cools H, Shaw M, Fillinger S, Terrado EM, Schnabel G, Mehl A & Oliver RP (2016) Proposal for a unified nomenclature for target site mutations associated with resistance to fungicides. *Pest Management Science* 8, 1449-59

- Mullins JGL, Parker JE, Cools HJ, Martel CM, Togawa RC, Lucas JA, Fraaije BA, Kelly DE & Kelly SL (2011). Molecular Modelling of the Emergence of Azole Resistance in *Mycosphaerella graminicola*. *PLoS. One* 6, 1-11 (e20973).

- Parker J, Warrilow A, Cools H, Fraaije BA, Lucas J, Rigdova K, Griffiths W, Kelly D & Kelly S (2013). Prothioconazole and prothioconazole-desthio activity against *Candida albicans* sterol 14-α demethylase (CaCYP51) *Applied and Environmental Microbiology* 79, 1639-1645.

- Parker JE, Warrilow AGS, Cools HJ, Martel CM, Nes WD, Fraaije BA, Lucas JA, Kelly DE & Kelly SL (2011). Prothioconazole binds to *Mycosphaerella graminicola* CYP51 by a different mechanism compared to other azole antifungals. *Applied and Environmental Microbiology* 77, 1460-1465.

- Rudd J, Antoniw J, Marshall R, Motteram J, Fraaije B & Hammond-Kosack K (2010). Identification and characterisation of *Mycosphaerella graminicola* secreted or surface-associated proteins with variable intragenic coding repeats. *Fungal Genetics and Biology* 47, 19-32.

- Yamashita M and Fraaije BA (2018) Non-target site SDHI resistance is present as standing genetic variation in field populations of *Zymoseptoria tritici*. *Pest Management Science* 74: 672-681

- Young C, Van den Bosch F, Fraaije BA, Burnett F, Knight S, Gosling P & Paveley N (2017) Strategies to delay the development of fungicide resistance: Alternation, mixtures or restricting the number of treatments? *Aspects of Applied Biology* 134; 37-43.

Conference proceedings/book chapters

- King K, Kirikyali N, West J & Fraaije BA (2017) Rapid LAMP assays to detect MgCYP51 and/or MgMFS1 overexpressing strains of *Zymoseptoria tritici* in leaf samples. In: Deising HB; Fraaije BA; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "*Modern Fungicides and Antifungal Compounds*", Vol. VIII, pp. 67-72. Deutsche Phytomedizinische Gesellschaft.

- Gutierrez-Alonso O, Hawkins N & Fraaije BA (2017) *In vitro* evolution of fluxapyroxad resistance in *Zymoseptoria tritici*. In: Deising HB; Fraaije BA; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "*Modern Fungicides and Antifungal Compounds*", Vol. VIII, pp. 113-114. Deutsche Phytomedizinische Gesellschaft.

- Kirikyali N, Diez P, Luo J, Hawkins N & Fraaije BA (2017) Azole and SDHI sensitivity status of *Zymoseptoria tritici* field populations sampled in France, Germany and the UK during 2015. In: Deising HB; Fraaije BA; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "*Modern Fungicides and Antifungal Compounds*", Vol. VIII, pp. 153-158. Deutsche Phytomedizinische Gesellschaft.

- Hawkins NJ & Fraaije BA (2017) Adaptive landscapes in fungicide resistance: fitness, epistasis, constraints and predictability. In: Deising HB; Fraaije BA; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "*Modern Fungicides and Antifungal Compounds*", Vol. VIII, pp. 217-221. Deutsche Phytomedizinische Gesellschaft

- Van Den Bosch, F., Fraaije, B. A., Oliver, R., Van Den Berg, F. and Paveley, N. 2015. The use of mathematical models to guide fungicide resistance management decisions. in: Hollomon, D. W. and Ishii, H. (ed.) *Fungicide resistance in plant pathogens* Springer, Tokyo. pp. 49-62

- Van Den Bosch, F., Paveley, N., Fraaije, B. A., Van Den Berg, F. and Oliver, R. 2015. Evidence-based resistance management: a review of existing evidence. in: Hollomon, D. W. and Ishii, H. (ed.) *Fungicide resistance in plant pathogens* Springer, Tokyo. pp. 63-76

- Fraaije BA, Atkins S, Luo J, Diez de la Fuente P & Hawkins NJ (2014) Evolution and spread of azole-insensitive *Zymoseptoria tritici* field isolates in the UK. Phytopathology 104 (S3): S3.41.

- Fraaije BA (2014) Use of loop-mediated isothermal amplification assays to detect azole-insensitive *CYP51*-overexpressing strains of *Zymoseptoria tritici*. *Phytopathology* 104 (S3): S3.41-42.

- Lucas JA & Fraaije BA (2014) Sustainable crop protection: the storm before the calm? *Proceedings Crop Protection in North Britain 2014*, 61-70.

- Hawkins NJ & Fraaije BA (2014) Sterol biosynthesis inhibitors: Modes of resistance and development of CYP51 mutations." In *Abstracts of papers of the American Chemical Society*. Vol. 248.

- Hawkins NJ, Cools HJ & Fraaije BA (2013) Mapping the adaptive landscape of *Mycosphaerella graminicola CYP51* under selection by azole fungicides. *Abstract book Congress of the European Society for Evolutionary Biology*, 1. Experimental Evolution on Emperical Fitness Landscapes, 19

- Fraaije BA, Atkins S, Luo J, de la Fuente PD, Hawkins NJ & Cools HJ (2013). Evolution and spread of *Mycosphaerella graminicola* isolates overexpressing *CYP51* or carrying different CYP51 variants in response to azole fungicide applications. *Acta Phytopathologica Sinica* 43 (suppl.), 144.

- Fraaije BA & Cools HJ (2013). Detection of azole insensitive *CYP51* overexpressing strains of Mycosphaerella graminicola using loop-mediated isothermal amplification (LAMP) assays. *Acta Phytopathologica Sinica* 43 (suppl.), 289.

- Fraaije BA (2013). Update on the evolution and selection for azole and Succinate DeHydrogenase Inhibitor (SDHI) fungicide insensitivity in UK *Mycosphaerella graminicola* populations. *Acta Phytopathologica Sinica* 43 (suppl.), 570.
- Hawkins NJ, Cools HJ & Fraaije BA (2013). *CYP51* and evolution of azole resistance. *Acta Phytopathologica Sinica* 43 (suppl.), 570.
- Cools HJ & Fraaije BA (2012) Resistance to azole fungicides in European populations of *Mycosphaerella graminicola*: Mechanisms and Management. *In: Fungicide resistance in Crop Protection: Risk and Management.* CABI. Wallingford, UK. pp 64-77.
- Cools HJ, Parker JE, Motteram J, Kelly DE, Lucas JA, Rudd JJ, Kelly SL & Fraaije BA (2011). Functional characterisation of *Mycosphaerella graminicola* sterol 14α-demethylase (MgCYP51) variants resistant to azole fungicides. In*: Modern Fungicides and Antifungal Compounds VI,* 123-128. 16th International Reinhardsbrunn Symposium, Friedrichroda, Germany.
- Clark WS, Fraaije BA, Lucas JA &. Cools HJ (2010). Septoria resistance and azole use 2010. *RRA Newsletter* 31.

Conference presentations
- Fraaije BA. Invited speaker at the International Symposium on Cereal Leaf Blights, University College Dublin (22 May 2019) 'Cultural management, fungicide resistance and epidemiology of cereal leaf blights; with a special focus on Septoria leaf blotch.
- Fraaije BA. Invited speaker at the 19th International Reinhardsbrunn Symposium in Friedrichroda, Germany (8 April 2019) 'SDHI sensitivity status of UK *Zymoseptoria tritici* and *Pyrenophora teres* field populations'.
- Fraaije BA. Invited speaker at the 2017 APS Annual meeting in San Antonio, Texas, USA (8 August 2017) 'Evolution of multi-fungicide resistance in cereal pathogens and impact on disease control'.
- Fraaije BA. Invited speaker at the 18th International Reinhardsbrunn Symposium in Friedrichroda, Germany (24 April 2016) 'Azole and SDHI sensitivity status of *Zymoseptoria tritici* field populations sampled in France, Germany and the UK during 2015
- Fraaije BA. Invited speaker at the 9th International Symposium on Septoria Diseases of Cereals in Paris, France (7 April 2016) 'The epidemiology, disease management and evolution of fungicide resistance in *Zymoseptoria tritici*'

- Fraaije BA. Invited speaker at 48*th* *Congresso Brasileiro de Fitopatologia* in São Pedro, Brazil (12 August 2015) 'Contrast between intensive fungicide use or sustainable integrated disease management for the emergence of fungicide resistance'
- Fraaije BA. Invited speaker at the Colloquium and Master Class on azole resistance in *Aspergillus fumigatus* 'Azole resistance in *Zymoseptoria tritici* and control strategies' in Amsterdam, The Netherlands (2-4 March 2015).
- Fraaije BA. 'Evolution and spread of azole-insensitive *Zymoseptoria tritici* field isolates in the UK', 2014 APS-CPS joint meeting. Minneapolis, Minnesota, USA, 12 August 2014.
- Fraaije BA. Invited speaker 'Update on the evolution and selection for azole and succinate dehydrogenase inhibitor (SDHI) fungicide insensitivity in UK *Mycosphaerella graminicola* populations. 10*th* International Conference of Plant Pathology (ICPP), Beijing, China, 25-30 August 2013.
- Fraaije BA. 'The use of azole and sdhi fungicides to control Septoria leaf blotch in the UK', Reinhardsbrunn Conference, Friedrichroda, Germany, 21-25 April 2013.
- Fraaije BA. Investigations into the molecular mechanisms responsible for the decline in sensitivity to DMI fungicides in *Mycosphaerella graminicola* populations' 2012 APS Annual meeting, Providence, RI, USA, 6 August 2012.
- Fraaije BA. Invited speaker 'CYP51 evolution and azole sensitivity in fungal pathogens of cereal crops' 11*th* Symposium on Cytochrome P450 Biodiversity and Biotechnology, Torino, Italy, 22-26 June 2012.
- Fraaije BA. 'Effects of DMI fungicides on selection of CYP51 variants in UK populations of *M. graminicola*'. Reinhardsbrunn Conference, Friedrichroda, Germany, 25-29 April 2010.

Conference posters
- Fraaije BA, Diez de la Fuente P, Atkins S & Hawkins NJ (2019) 'Evolution and spread of SDHI resistance in UK field populations of *Zymoseptoria tritici*' at APS Annual meeting 'Plant Health', Cleveland (USA) (3-7 August).
- Fraaije BA (2018) 'Evolution of fungicide resistance in UK field populations of *Zymoseptoria tritici*' at ICPP2018 'Plant health in a global Economy' Boston (USA) (29 July – 3 August).
- Fraaije BA, Atkins S, Luo J, Diez de la Fuente P & Hawkins NJ (2014) Evolution and spread of azole-insensitive *Zymoseptoria tritici* field isolates in the UK. Poster APS Conference, Minneapolis, USA.

- Fraaije BA (2014) 'Use of loop-mediated isothermal amplification assays to detect azole-insensitive *CYP51*-overexpressing strains of *Zymoseptoria tritici*' at APS Annual meeting, Minneapolis, USA.
- Fraaije BA, Atkins S, Luo J, de la Fuente PD, Hawkins HJ & Cools HJ. (2013) Mapping the adaptive landscape of *Mycosphaerella graminicola* CYP51 under selection of azole fungicides. Poster International Conference of Plant Pathology, Beijing, China.

Knowledge transfer: presentations and workshops

- Fraaije BA. Regular presentations of project results at FRAG-UK 6 months interval meetings
- Data/slides provided for AHDB Agronomists' Conferences (see Stuart Knight presenting the latest fungicide performance data at the 2018 AHDB Agronomists' Conference (https://ahdb.org.uk/knowledge-library/fungicide-performance))
- Fraaije BA. Invited speaker at the AICC conference (Towcester, 11/01/17) Presentation 'Evolution of fungicide resistance in *Z. tritici*' in the session 'Dealing with resistance using fewer tools; what are the implications and how can they be overcome?
- Fraaije BA. Invited speaker at the Dow AgroSciences Inatreq launch meeting in Indianapolis, USA (10 February 2016) 'Evolution of fungicide resistance in cereal pathogens: *Zymoseptoria tritici*'
- Fraaije BA. Invited speaker at 'Resistance monitoring of key arable diseases in the UK' at Raddisson Blu Hotel, London Stansted Airport (24 November 2014). Presentation on MBCs (Light leaf spot (LSS), DMIs (Rhynchosporium, LLS and Septoria leaf blotch (SLB)) and SDHIs (SLB).
- Fraaije BA. Invited speaker 'Evolution of azole resistance in the cereal pathogens *Fusarium graminearum*, *Rhynchosporium commune* and *Mycosphaerella graminicola*', French agronomists meeting organised by Bayer CropScience, Burnham Beeches hotel, Burnham, UK, 25 June 2013.
- Fraaije BA. Invited speaker 'Resistance and shifts in fungicides' at Frontier's Agronomy Winter conference, University of Warwick, 5-6 December 2012.
- Fraaije BA. Organiser and speaker 'Selection of CYP51 variants in UK field populations of *Mycosphaerella graminicola*: a historic perspective', EPPO workshop 'Azole fungicides and control of Septoria leaf blotch', Rothamsted, Harpenden, 8 December 2010.

- Fraaije BA. 'What can the archives tell us about changes in pathogen incidence? PPM day, Rothamsted, 18 November 2010.
- Fraaije BA. Invited speaker 'Exploiting Rothamsted's classical experiments: impact of agricultural practices on microbes (emphasis on Evolution of fungicide resistance in *Mycosphaerella graminicola*), 12 August 2010, Novozymes, Salem, USA.

Articles in the farming press

- *Crop Production Magazine* 2017 'Making sense of resistance' http://www.cpm-magazine.co.uk/wp-content/uploads/2017/02/DiseaseMar17.pdf
- *Crop Production Magazine* 2016 From Theory to field 'Quest underway for resilient strategies' https://cereals.ahdb.org.uk/media/903800/T2F-April-2016-–-SDHI-resistance-strategies.pdf
- *Farmers Weekly*, 12 February 2016 'First sign of resistance to SDHI fungicides found in the UK' https://www.fwi.co.uk/arable/first-sign-resistance-sdhi-fungicides-found-uk
- *Farmers Guardian*, 12 February 2016. SDHI fungicide sensitivity shift detected in UK septoria isolates https://www.fginsight.com/news/sdhi-fungicide-sensitivity-shift-detected-in-uk-septoria-isolates-9974
- *Farmers Guide*, 12 February 2016. SDHI fungicide sensitivity shift detected in two UK septoria isolates https://www.farmersguide.co.uk/2016/02/sdhi-fungicide-sensitivity-shift-detected-in-two-uk-septoria-isolates/
- *FRAG-UK statement* May 2016. https://cereals.ahdb.org.uk/media/907922/FRAG-SDHI-Statement-May-2016.pdf
- *Farming on-line*, 15 April 2014 'Protecting azole and SDHI efficacy' https://www.farmersguide.co.uk/2014/04/protecting-azole-and-sdhi-efficacy/
- *Farmers Weekly*, 15 April 2014 'Growers need to act to protect fungicide efficacy' https://www.fwi.co.uk/arable/growers-need-to-act-to-protect-fungicide-efficacy

Press releases, newsletters and topic sheets

- *FRAG-UK statement* May 2016. https://cereals.ahdb.org.uk/media/907922/FRAG-SDHI-Statement-May-2016.pdf (appendix A)
- SDHI LINK project – key messages for press release. Messages from the project were presented and discussed by the UK Fungicide Resistance Action Group (FRAG) on 26 March 2014 (appendix B)

- HGCA topic sheet 113 '*Septoria tritici* in winter wheat' spring 2012 https://cereals.ahdb.org.uk/media/178045/ts113_septoria_tritici_in_winter_wheat .pdf

Appendix A

The Succinate Dehydrogenase Inhibitor fungicides (SDHIs) are broad spectrum and highly effective against barley and wheat diseases. The SDHIs bring improvements in disease control and yield compared to many older fungicides.

Resistance to fungicides can evolve rapidly where resistance is conferred by a simple mutation, particularly where this does not confer a fitness penalty on mutated individuals. Resistance is less likely and/or slower to develop where multiple genes are involved.

FRAG-UK considers that the SDHI fungicides are at a medium/high risk of resistance development in cereal pathogens.

In 2012, extensive monitoring detected two isolates of septoria (*Zymoseptoria tritici*) (France and UK) with sensitivities outside the baseline range. Resistance factors were very low and field performance was not affected.

From 2013 to 2014, further isolates of septoria with mutations conferring low levels of reduced sensitivity to SDHI fungicides were detected sporadically throughout Europe.

In December 2015, Teagasc confirmed the detection in the Republic of Ireland of field isolates of septoria at low frequency with reduced sensitivity to the SDHI group of fungicides. Some of these isolates had a mutation (C-H152R) in the SDHI target site that had not been detected in field isolates previously. These Irish isolates are approximately 100 times less sensitive in laboratory tests than a broad range of field isolates collected previously.

At the end of 2015, Rothamsted Research reported low frequencies of SDHI insensitive strains in UK field populations. Four different mutations, B-T268I, C-T79N, C-N86S and CI161S, and efflux pump (MgMFS1) overexpressing strains contributed to the SDHI insensitive phenotype but resistance factors in these isolates remain low.

In May 2016, Rothamsted Research reported results from further isolates collected late in the 2015 season. Three of these carry the same mutation (C-H152R), as reported from Ireland, while one strain carries both C-N86S and D-D129E mutations. These new isolates were tested in glasshouse trials on seedlings. The most insensitive one was unaffected by full dose straight SDHI and the other causing septoria symptoms at a quarter or half rate SDHI.

More research is needed to determine how these findings impact on disease control in the field. Although field performance from SDHIs is still anticipated to be good in 2016, strong stewardship is needed to prevent a rapid increase in the frequency of such isolates in the septoria population.

In view of these latest findings, it is crucial that fungicide programmes on wheat and barley adhere strictly to the guidelines on resistance management (detailed below) so declines in sensitivity to either DMI or SDHI fungicides are not accelerated through high-risk practices. The industry collectively and individually can and must act to steward these products.

Guidelines on resistance management

All effective fungicides exert a selection pressure on pathogen populations and carry a risk of resistance. This risk can be modified and reduced by either mixing or alternating with fungicides with an alternative mode of action, or by reducing the number of applications or dose of the fungicide. Not enough is known about the relative impact of these strategies to be able to prioritise one over any other.

To maintain the efficacy of the SDHI fungicides against medium and high-risk pathogens in cereals, such as Z. tritici in wheat and Pyrenophora teres and Ramularia collo-cygni in barley, FRAG-UK recommend the

following measures to reduce the risk of fungicide resistance development and extend product life. These guidelines apply to both spring and winter cereals:

Scenario 1 – SDHI foliar applications 1. Follow the statutory requirement to limit the number of applications to two SDHI fungicide containing sprays. 2. Always use SDHI fungicides in mixture with at least one fungicide from an alternative mode of action group which has comparable efficacy against the target pathogen(s). 3. Tank mixing two SDHI fungicides is not an anti-resistance strategy. In any tank mix, the SDHI should be applied in a balanced mixture with at least one fungicide with comparable efficacy against the target pathogens from an alternative mode of action group.

Scenario 2 – SDHI seed treatments with no efficacy against foliar pathogens 1. These do not count towards the statutory limit of two foliar SDHI applications; advice would therefore be to apply any subsequent foliar SDHI applications as described in Scenario 1 above. 2. The SDHI seed treatment should be co-formulated with a fungicide with an alternative mode of action to reduce selection pressure on seed-borne pathogens.

Scenario 3 – SDHI seed treatments with efficacy against foliar pathogens 1. There are currently no SDHI seed treatments with efficacy against foliar pathogens approved in the UK. 2. Should any such seed treatments be approved, FRAG-UK would advise that these should count as one of the statutory limit of two SDHI applications to a crop. It would therefore become necessary that growers keep records of seed treatments applied.

ICM statement In order to reduce disease risk and the selective pressure on fungicides, integrated crop protection measures (ICM) should be used, such as the use of resistant varieties. Seed should be tested to identify the seed-borne pathogens present.

ALWAYS CONSULT PRODUCT LABELS BEFORE USE

Examples of current SDHI fungicides Product name and active ingredients (SDHI in bold) Adexar **fluxapyroxad** + epoxiconazole Aviator Xpro **bixafen** + prothioconazole Bontima **isopyrazam** + cyprodinil Ceriax **fluxapyroxad** + epoxiconazole + pyraclostrobin Enterprise **boscalid** + epoxiconazole Nebula **boscalid** + epoxiconazole + pyraclostrobin Imtrex **fluxapyroxad** Librax **fluxapyroxad** + metconazole Keystone **isopyrazam** + epoxiconazole Siltra Xpro **bixafen** + prothioconazole Tracker **boscalid** + epoxiconazole Treoris **penthiopyrad** + chlorothalonil Vertisan **penthiopyrad** Zulu **isopyrazam**

About the Fungicide Resistance Action Group – UK

Aims and Objectives To gather and interpret information on fungicide resistance and its management and arrive at a United Kingdom (UK) consensus view.

To promote practical guidance on the status and management of fungicide resistance in the UK.

To indicate areas where Research and Development are required.

To provide an interface between industry's Fungicide Resistance Action Committee (FRAC) official bodies and independent research organisations.

To produce, publish and promote educational material that will assist in the understanding of and reduce the incidence of resistance in plant pathogens.

Current Steering Group Members:
Dr Fiona Burnett (Chair) Scotland's Rural College (SRUC) – Email: Fiona.Burnett@sruc.ac.uk   Mr Paul Ashby (Secretary) CRD, HSE Mr M Ashworth DuPont Dr J Brown JIC Mr B Clark NIAB-TAG Mr R Clayton AHDB Dr L Cooke Queen's University, Belfast Mr D Ellerton AIC Dr Kerry Macguire Bayer CropScience Dr P Fogg ADAMA Dr B Fraaije RRES Dr P Gosling AHDB Mr A Horgan CERTIS Mr A Jones Chemtura Dr G Kemmitt FRAC Mr S Leak Belchim Mr B McKeown BASF Dr G M McPherson STC Dr N Paveley ADAS Mr J Sellars Dow Mr J Tatnell Syngenta Mr M Thompson AICC Dr J Turner FERA Dr G Young AFBI

Appendix B

SDHI LINK project – key messages for press release.
These messages from the project were presented and discussed by the UK Fungicide Resistance Action Group (FRAG) on 26 March 2014.

There is good evidence that:
- The shift in azole performance against septoria continues (HGCA Fungicide Performance data)
- The UK has a more pronounced decline in azole performance than elsewhere in Europe, where disease pressure is lower and azole use is less intense.
- Future azole performance in the UK depends on how we use them.
- The project data shows that selection for azole resistant strains is driven mainly by the number of applications (more sprays = more exposure of the pathogen to the mode of action = more selection).
- Azole use at T0 (for rust) and T3 (for ear blight), in addition to their use at the 'core' T1 and T2 timings, increases selection of insensitive septoria strains. Any additional sprays will only increase this selection pressure i.e. T1.5 or T4 sprays
- The small incremental shifts in azole sensitivity are only weakly affected by dose.
- Whereas selection for SDHI resistance likely to be increased both by dose and by number of treatments.
- Mixtures reduce selection for resistance.
- Doses of mixture components can be optimised to reduce selection.
- The azole sensitivity situation remains fluid, is shifted by even single azole applications and there is variability in the occurrence and frequency of mutations found at sites within the UK.
- The project has developed screening methods needed to rapidly screen for the most high-risk SDHI and azole mutations.

The project has provided evidence to support strategies for fungicide usage that reduce the risk of resistance. The dual aim of these strategies is to:
Use azoles to protect SDHIs from resistance, but minimise selection of azole resistant strains
Use SDHIs to protect azoles from resistance, but minimise selection of SDHI resistant strains
This is a challenging task, which has to balance the resistance risks against azoles and the SDHIs (the latter remain at high resistance risk) and the need for effective control. The guidance from the SDHI LINK project is to:
- Use the minimum number of azole applications required to achieve effective control. Consider alternatives to azoles where the main target at T0 is septoria protection (multi-sites are effective in this situation) or rusts (stobilurins remain highly effective against rusts). Avoid the use of additional or split sprays (i.e. T1.5, T4).
- The aim is to reduce the number of azole treatments per crop.
- Use mixtures of modes of action effective against septoria.
- Mix components as follows:
- Maximise control from multi-site acting fungicides (which are at low resistance risk) throughout the programme.
- Use robust doses of azoles at core timings. This will help to achieve effective control, maximise protection of the SDHI components in mixtures and minimise the need for additional /split sprays.
- Use the minimum SDHI dose and number of treatments required to achieve effective control. Partnering with a robust dose of azole will reduce SDHI resistance risk without markedly increasing selection for azole resistance.