University of Reading

Fungicide-resistance management tactics: impacts on *Zymoseptoria tritici* populations

Thesis submitted in fulfillment of the Degree of Doctor of Philosophy

School of Agriculture, Policy and Development

Hilda Dooley

January 2015

Abstract

Azoles and Succinate Dehydrogenase Inhibitors (SDHIs) are the main fungicides available for septoria tritici blotch control, causal agent *Zymoseptoria tritici*. Decline in azole sensitivity, in combination with European legislation, poses a threat to wheat production in Ireland.

Azole fungicides select *CYP51* mutations differentially; it was hypothesised that using combinations of azoles could be an effective anti-resistance tool. Naturally inoculated field experiments were carried out in order to understand the impacts of using combinations of azoles, epoxiconazole and metconazole, on azole sensitivity. Approximately 3700 isolates were isolated and their sensitivity to both azoles analysed. Findings showed that limiting the number of applications, by alternating each fungicide, slowed selection for reduced azole sensitivity. Limiting azole use by reducing doses did not reduce selection for decreased azole sensitivity. Although not complete, cross-resistance was observed between the two azoles, which will lead to general reduction in azole sensitivity.

A sub-selection of isolates from each treatment at each location were analysed for changes in the *CYP51* gene. Sequence analysis identified 49 combinations of mutations in the *CYP51* gene, and three different inserts in the *CYP51* promoter. Intragenic recombination also featured in these populations.

Baseline studies of five new SDHIs were carried out on 209 naturally infected, non-SDHI-treated isolates. With the exception of fluopyram, cross-resistance was apparent between the SDHIs. Analysis of 2300 isolates found that when compared to the solo products, mixing the SDHI isopyrazam and the azole epoxiconazole increased epoxiconazole sensitivity, but had no apparent effect on isopyrazam sensitivity. SDHI resistance-conferring mutations were absent in the baseline and experimental isolates.

As long as azoles are used, *Z. tritici* populations will continue to evolve towards resistance. Combining different modes-of-action, SDHIs and multi-sites, with azoles will relieve some of that selective pressure. To get the best out of available fungicides, they should be used in combination with host resistance and good crop management practices.

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Signed …….

Date……..

Acknowledgements

This PhD was funded by Teagasc under its Walsh fellow scheme. The Walsh fellow scheme also funded an invaluable three month study trip to the phytopathology lab in ETH, Zurich.

I would like to take this opportunity to thank a few people who supported me throughout my PhD studies.

To all Teagasc research officers, technicians and farm staff who helped me whenever I needed it. Special thanks to Jim Grace and Liz Glynn for all the help with field experiments and for helping to find data which went rogue at times. Thanks Jeanne Mehenni-Ciz for the help in the lab.

To the undergraduate students who helped me through the long day's disease assessing and isolating, thank you Aaron Mullins, Gillian Darby, Clair Jouan and Aurelien Lepenettier.

To all of the other PhD students at Teagasc, for keeping me sane during stressful times, and for supplying the gossip that kept me smiling, thank you.

To my supervisors: for furnishing me with the skills needed to complete such a mammoth task as a PhD; for allowing me to develop as a researcher at my own pace; for such graceful reactions to my tears of frustration; and especially for almost never disagreeing with each other, thank you Steven Kildea, Mike Shaw and John Spink. At this time I must also thank Bruce McDonald (and everyone at ETH) for welcoming me into the phytopathology lab at ETH, and for facilitating the collaboration with Patrick Brunner who advised and assisted with analysis and discussion points for Chapter 3. Bruce and Patrick, thank you.

To all of my family and friends who have accepted without question my virtual absence for the last two years. To my parents, who instilled in me the work ethic that got me through this PhD, and who are still working hard to keep all of their children, grandchildren and friends happy, thank you.

Last, but by no means least, to Mike, for putting up with me over the last three years, I may have cracked by now if it weren't for you. Thank you.

Table of Contents

List of Figures

List of Tables

Abbreviations

ABC: ATP-binding cassette

a.i.: active ingredient

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

bp: base pair

cm: centimetre

DMI: Demethylation inhibitors

DMSO: dimethyl sulfoxide

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleotide Triphosphates

FRAC: Fungicide Resistance Action Committee

FRAG-UK: Fungicide Resistance Action Group

GS: growth stage

ha: hectare

IPM: Integrated pest management

L: litre

MBC: Methylbenzimidazole carbamates

MDR: multiple drug resistance

MFS: Major facilitator superfamily

mg: milligrams

min: minute

ml: millilitres

ML: maximum likelihood

N: nitrogen

Ng: nanograms

nM: nano molar

nm: nanometres

PCA: principal components analysis

PCR: polymerase chain reaction

PDB: potato dextrose broth

PGA: potato glucose agar

QoI: Quinone outside Inhibitors

RBP: recombination break point

REML: Restricted maximum likelihood

s: seconds

SDHI: Succinate dehydrogenase inhibitors

sqrt: square root transformed

STB: Septoria tritici blotch

t: tonnes

t/ha: tonnes per hectare

TM: trade mark

U: unit

UK: United Kingdom

µl: microliter

µM: micromolar

UV: ultra violet

WT: wild type

°C: degrees Celsius

Chapter 1: Introduction

1.1 Preface

Wheat is the most important cereal grown in the Northern Hemisphere (Oerke & Dehne, 2004), and is the second most important food crop after rice in developing countries (http://www.wheatinitiative.org/). The worldwide average yield of wheat is approx. three t/ha (Hawkesford *et al*., 2013), but Irish winter wheat crops produce some of the highest yields per hectare in the world (Jess *et al*., 2014), averaging over eight t/ha between 2007-2010 (J. Spink, personal communication). Ireland's temperate climate with high rainfall during the growing season complements wheat (*Triticum aestivum*) production, but is also conducive for the growth of *Zymoseptoria tritici*, the causal agent of Septoria tritici blotch (STB). Although a number of diseases affect wheat yields, STB is the main disease of winter wheat in Ireland and many other wheat growing regions throughout the world (Viljanen-Rollinson *et al*., 2005, Fraaije *et al*., 2012, Cools & Fraaije, 2013). Septoria tritici blotch is characterised by irregular necrotic lesions interspersed with small black fruiting bodies (pycnidia) on the leaves (Palmer & Skinner, 2002) and stem (Ponomarenka *et al*., 2011). It is these lesions that reduce the green leaf area of the plant and which, particularly if present on the upper leaves during grain filling, can reduce yield (Gooding *et al*., 2000). Burke and Dunne (2008) recorded yield reductions of up to 50% resulting from STB under Irish growing conditions. Such yield reductions make the cultivation of wheat economically un-viable. Given the importance of wheat globally and locally, and considering the potential losses due to STB, adequate control of *Z. tritici* is important for the continuation of wheat cultivation in Ireland and Europe.

1.2 Biology of *Zymoseptoria tritici*

Zymoseptoria tritici (Desm.) Quaedvlieg & Crous is part of the *Mycosphaerella* complex which contains a number of different families and genera (Quaedvlieg *et al*., 2011). Formerly known as *Mycosphaerella graminicola* ((Fuckel) Schröter in Cohn), Quaedvlieg *et al*. (2011) designated *Septoria*-like species with graminicolous hosts to the *Zymoseptoria* genus. Morphologically, *Zymoseptoria* species have a yeast-like growth form in culture, and types of conidia unlike those in the *Septoria* genus (Quaedvlieg *et al*., 2011). *Zymoseptoria tritici* is a haploid (with a transient diploid stage (Whittenberg *et al*., 2009)), heterothallic ascomycete (Kema *et al*., 1996), which has a hemibiotrophic lifecycle and reproduces both sexually (teleomorph) and asexually (anamorph). It has a polycyclic lifecycle within wheat crops (Henze *et al*., 2007) and undergoes multiple sexual (Kema *et al*., 1996) and asexual (Shaw & Royle, 1993) lifecycles throughout the growing season. The lifecycle of STB begins soon after the emergence of the wheat crop. Ascospores, which are the sexual progeny, are capable of disseminating over distances of between 10-200 kilometres (Linde *et al*., 2002) . They are the primary source of inoculum (Shaw & Royle, 1989), as well as contributors to the within season spread of disease (Kema *et al*., 1996). They remain in perithecia in the stubble of previous crops (Palmer & Skinner, 2002) until after periods of moisture and subsequent fluctuations in relative humidity (Ponomarenka *et al*., 2011) when spores are ejected into the air and land on a wheat host. Spores then germinate on the leaf surface and hyphae gain entry to the leaf through the stomata (Ponomarenka *et al*., 2011) where they grow inter-cellularly for a period of up to two weeks without any apparent damage to the host (Goodwin *et al*., 2011). The pathogen then switches from a biotroph to a necrotroph and causes host cell collapse which causes the necrotic lesions form. The long latent period (14-28 days) and the ability to switch from biotrophy to nectrotrophy facilitates the evasion of host defence systems by *Z. tritici* (Goodwin *et al*., 2011). Pycnidia form around the stomata within the

necrotic areas and exude conidia (Ponomarenko *et al.*, 2011). These conidia, which cause the secondary and most of the subsequent infections (Gladders *et al*., 2001), are discharged after rain and high humidity events (Shaner & Finney, 1976). Additionally, conidia are tolerant of low levels of humidity/moisture (Shaw, 1991), surviving desiccation and allowing the pathogen to withstand fluctuations in levels of humidity (Gough & Lee, 1985).

1.3 Controlling *Zymoseptoria tritici*

Septoria tritici blotch can be controlled through a combination of measures including good crop management practices, varietal resistance, and chemical control.

1.3.1 Crop management

The most basic agronomic practice for general control of cereal diseases is crop rotation. It helps improve soil structure and nutrient management, which both serve to strengthen the crop, and it removes the host from the immediate vicinity of lingering inoculum. However, while crop rotation has obvious benefits for controlling soil borne diseases such as take-all (Anon, 2014c), diseases which spread in the air such as STB will find their host over a wider area. Because of soil conservation and potential savings in crop establishment costs, minimum tillage (min-till) practices have gained popularity in recent years. Not much research has been done on the effects of min-till on STB epidemics, however, considering that ascospores on wheat stubble provide the biggest proportion of primary inoculum (Suffert *et al*., 2011) it could be suggested that min-till is likely to increase the level of primary inoculum early in the season (Sept-April, (Duvivier *et al*., 2013)), which depending on environmental conditions may have a knock-on effect on disease severity on important leaf layers. Conversely, the presence of microflora in the undisturbed soil system may reduce transfer of disease to the next crop or encourage early defences. Later sowing reduces the host availability early in the season, decreasing the ‘green-bridge’ between crops and potentially

reducing the carry-over of inoculum between crops. Gladders *et al*. (2001) established a link between later sowing and reduced disease severity later in the growing season. They showed that disease risk was significantly reduced by October sowing compared to September sowing, and that thresholds were rarely reached after a November sowing time (in the UK). Increased severity of STB under high nitrogen (N) fertilisation has been demonstrated (Broscious *et al*., 1984, Leitch & Jenkins, 1995, Simón *et al*., 2003), but effects may depend on whether the environmental conditions are conducive to N uptake and disease progress (Simón *et al*., 2003). Hence, good management of soil N could be a factor in reducing STB severity. It is possible that managing the crop architecture through controlling seed rate has an effect on STB development, however, results are variable (Baccar *et al*., 2011). Baccar *et al*. (2011) found no differences in Septoria epidemics between seeding rates tested but Broscious *et al*. (1984) established that in some instances where higher seeding rates were used, significantly higher levels of STB were observed. Higher seeding rates lead to a closed canopy, possibly allowing easy transfer of spores horizontally and vertically within the crop and providing a suitable microclimate for the development of the disease (Tompkins *et al*., 1993). On the other hand, a more sparse open canopy would facilitate higher rain splash (Eyal, 1981) and easy spread of conidia up the canopy. Low N rates, late sowing and low seeding rates together may help to reduce STB incidence. Unfortunately, many of these practices are also likely to reduce yield (Green & Ivins, 1985), resulting in few growers using them.

1.3.2 Host resistance

In addition to good crop management, host resistance can play a role in STB control. Miedaner *et al*. (2013) argue that breeding for resistance to STB is possibly the most environmentally sound method of control, however development of STB host resistance is hard because resistance has complex inheritance (Zhan *et al*., 1998). Host resistance to STB

can be based on a single gene resistance, but is usually controlled by many loci each with a small effect size, i.e. polygenic resistance (Miedaner *et al.*, 2013), which reduces the risk of being overcome by a single resistant *Z. tritici* isolate. Host resistance is available for STB, but good host resistance often comes with a trade-off, for example susceptibility to other pathogens or reduced yields (Brown, 2002). Some of the higher yielding cultivars which are typically grown in Ireland come with low STB resistance, often with resistance ratings of 4 to 5 on a scale of 1-9, where 1 is susceptible and 9 is resistant (Anon, 2013c), which alone is not enough to maintain sufficient STB control, especially under high disease pressure. Gigot *et al.* (2013) however demonstrated that mixing cultivars, at a 3:1 ratio of resistant:susceptible, had the effect of reducing the level of sporulation on the susceptible host, compared to the pure stand. Even though that result was seen under low-medium levels of STB disease pressure and crops in Ireland are often under high disease pressure, this practice could contribute towards reducing STB levels at the same time as utilising the higher yielding potential of the susceptible cultivars. While field resistance to some of the major fungicide groups has renewed the drive to look for durable, effective host resistance (Arraiano *et al.*, 2009), current varietal resistance in the wheat-*Z. tritici* pathosystem is limited in its effectiveness.

1.3.3 Fungicides

The application of fungicides has been shown to contribute substantially to the yield of wheat (Blake *et al.*, 2011, Dunne *et al.*, 2008) and intensive cereal production has come to rely on chemical fungicides to secure yields in high disease pressure situations (Anon, 2014c). Cereal fungicides are applied as either foliar or seed treatments to protect against a range of fungal pathogens, including *Z. tritici*. While not targeting *Z. tritici* specifically, some researchers have demonstrated that seed treatments do have an effect on STB. Christ and Frank (1989) found that STB severity was reduced further in plots treated with a foliar and

seed treatments, compared to the foliar treatment alone. Dinoor (1977) found that a seed treatment including thiabendazole contributed a moderate level of protection from STB, but Shtienberg (1992) saw no such effect. Quérou *et al*. (1998) demonstrated that even though most of the triticonazole seed treatment was translocated to the roots of the wheat plant, some of the active ingredient reached the shoots also. Sundin *et al*. (1999) showed that triadimenol and difenconazole both suppressed sporulation levels of Septoria for up to 6.5 weeks after sowing.

Foliar fungicides, on the other hand, are used specifically to target STB and other foliar pathogens. The top three leaves of the wheat plant, which make the most important contribution to yield (Shaw & Royle, 1989), are best protected by applying foliar fungicides at precise timings (Paveley *et al*., 2000). The first main application should take place as soon as leaf 3 is fully emerged (approx. GS 32, (Zadoks *et al*., 1974)), and aims to give full protection to leaf 3 and some protection to leaf 2. The second main treatment, applied when the flag leaf is just fully emerged (approx. GS 39 (Zadoks *et al*., 1974)), aims to eradicate disease on leaf two and protect the flag leaf (Sylvester-Bradley *et al*., 2008). In situations where sprays are poorly timed, treated crops can suffer as much disease as untreated crops (Thomas, 1986 In: Cook *et al*. (1999)), so getting the timing of application right is important to ensure good control.

The rapid development of the fungicide market from the 1950s saw the introduction of many new active ingredients. The multi-site fungicides folpet and chlorothalonol were introduced in the 1950's and 60's (Russell, 2005). They are broad spectrum, contact fungicides (where the fungicide is not taken into the plant, and protects only the area where the fungicide lands), and act preventatively to impede spore germination (Leroux *et al*., 2005). While initially used for the control of diseases of fruit, veg and glasshouse crops, they are now primarily used for the control of STB. Active ingredients from the methyl

benzimidazole carbamate (MBC) group of fungicides were amongst the earliest (introduced in the late 1960s) systemic fungicides (where the fungicide is taken into the plant and transported around the plant in the transpiration stream (Russell, 2005)) to be used for the control of wheat diseases such as *Puccinia* spp., *Septoria* spp., and *Fusarium* spp. (Russell, 2005). Griffin and Fisher (1985) found that in the UK in 1981 the pressure from *S. tritici* was particularly high and, at that time, chemistries from the MBC group of fungicides were widely used for STB control. Demethylation inhibitors (DMIs), were introduced to the market from early 1970s, and grew to become a very important group of fungicides for many crops (Russell, 2005). DMIs are broad-spectrum, are mostly systemic and exhibit eradicant/curative activities (Leroux *et al.*, 2005). The azoles, largely represented by triazoles but also imidazoles, are the main chemical group within the DMI class. Indeed, for the control of STB, the DMIs were the main group until the introduction of the Quinone outside Inhibitors (QoIs). Strobilurins, initially azoxystrobin, from the QoIs were introduced in the 1990s (Fraaije *et al*., 2005) and became the main group of fungicides for control of cereal pathogens. This very effective group of systemic fungicides provided protectant and eradicant activity (Russell, 2005), with the addition of contribution to delayed senescence of the flag leaves (Ruske *et al*., 2003). Introduced in the mid-1960s, the carboxamide, carboxin, was used against rusts, bunts and smuts in cereals (Pasche *et al*., 2005). In more recent years, second generation carboxamides, commonly referred to as succinate dehydrogenase inhibitors (SDHIs), have been introduced. The first of the new SDHIs, boscalid, came on the market in 2005 (Fraaije *et al*., 2012), and between 2010 and the present (2014) five new SDHIs have been introduced for control of cereal diseases (Walter, 2011). The SDHIs exhibit largely protectant with some curative activity. Currently, the three main groups of fungicides available for STB control are the multi-site inhibitors, DMIs and SDHIs.

Effective fungicide use has the associated disadvantage of driving selection for resistant strains, leaving some fungicides less effective, or in extreme cases totally ineffective. The limited choice of fungicides available for use on cereals already places severe pressure on winter wheat production, and if any of the available fungicides for STB were to succumb to resistance, this would exacerbate the situation. On top of that, recent changes in European regulations on fungicides means that some of the fungicides which are available may be removed from the market in the near future, specifically the azoles and multi-sites (Blake *et al*., 2011, Jess *et al*., 2014). If azoles were to be removed from the market, it has been estimated that the drop in production of wheat in Europe would amount to 18.6 million t by 2020 (Di Tullio *et al*., 2012), which would have a knock on effect on worldwide markets (Jess *et al*., 2014).

1.4 Resistance evolution

1.4.1 Fungicide resistance

Fungicide resistance is defined as occurring when a fungal pathogen can survive and reproduce in the presence of a fungicide (Anderson, 2005). This is an acquired resistance that occurs after a period of exposure of the pathogen to a fungicide: the pathogen population which was sensitive to the fungicide at the time of introduction has become less sensitive over time and is no longer controlled adequately (Brent & Hollomon, 2007). Pathogens differ in their levels of risk of developing resistance depending on; the lifecycle of the pathogen - a pathogen with a short generation time and large number of propagules will be at a greater risk of resistance than one with a longer generation time and fewer propagules; and the inherent properties of the fungicide - a fungicide which targets multiple genes will be at a lower risk of resistance that one which targets a single gene (Brent & Hollomon, 2007). Resistance can develop as qualitative or quantitative resistance. Qualitative resistance, also known as single

gene or major gene resistance, happens when loss of efficacy is brought about by a single mutation in the target gene. Quantitative resistance, also known as multiple gene resistance, occurs when a gradual reduction in sensitivity is brought about by the development of many individual genetic changes, such as mutations in the target gene or over-expression of the target gene. Different terminology is used when qualifying resistance, i.e. if a strain is labelled laboratory resistant (resistance found in strains in controlled laboratory conditions) that does not automatically mean that those strains are field resistant (where a fungicide has a reduced level of control of the those strains in the field), and the presence of field resistant strains does not automatically mean that practical resistance (where total loss of efficacy of a fungicide is observed in the field) is present or imminent (Brent & Hollomon, 2007).

1.4.2 History of fungicide resistance in *Zymoseptoria tritici* isolates

The multi-site inhibitors which have been available since the 1950s and 60s (Russell, 2005) have not declined in efficacy since their introduction. Additionally, they have not been affected by resistance in *Z. tritici*, reflecting their low risk of resistance status (Brent & Hollomon, 2007). The systematic change in use from more general toxins such as the multi-sites to safer target-site-specific fungicides increased the risk of resistance occurring (Clark, 2006a). The first major development of resistance in *Z. tritici* to affect fungicide efficacy involved the methylbenzimidazole carbamate (MBC) group of fungicides, and occurred by 1984 (Griffin & Fisher, 1985). Brought about by a single genetic change (Fraaije *et al*., 2005), MBC resistance is still present in recent *Z. tritici* populations even though MBC fungicides have not been widely applied to wheat for over 20 years (O'Sullivan, 2009); evidence that it carries no significant fitness costs. After registration for use on cereals, the strobilurins quickly became key fungicides for cereal production (Russell, 2005). However, strains with major-gene resistance to the QoIs were found in *Z. tritici* populations in the UK and Ireland in 2002 (Fraaije *et al*., 2003), after which resistance developed rapidly in the

population. QoI resistance in *Z. tritici* is now complete, and fungicides from this group are no longer recommended for STB control. They are however still effective against other diseases such as *Puccinia* sp. in wheat (Oxley *et al*., 2014), and *Rhyncosporium commune* in barley (Gosling *et al*., 2014) and so still have a place in cereal disease control programmes. Additionally, they also have a greening effect on the plant (Anon, 2014c), so may be of use to help maintain green leaf area at important yield forming stages of the crop.

Like the MBCs and the QoIs (Fraaije *et al*., 2005), *Z. tritici* resistance to the SDHIs is purported to be due to monogenic or qualitative resistance. But, while the single-site mechanisms for SDHI resistance have been elucidated through lab experiments (Fraaije *et al*., 2012, Scalliet *et al*., 2012), a few field isolates with mutations conferring reduced sensitivity have been detected (Anon, 2014a). Even so, SDHI resistant *Z tritici* isolates have yet to be found in the field. FRAG rate the risk of *Z. tritici* resistance to SDHIs as medium-high, but because of the risk of single-site resistance occurring in SDHIs, other workers considered *Z. tritici* to be at a high-risk of resistance (Fraaije *et al*., 2012).

Resistance to azoles has been described as a polygenic trait (Stergiopoulos *et al*., 2003), or quantitative resistance, which develops in a gradual stepwise progression, and *Z. tritici* is thought to be at a medium risk of resistance to azoles (Brent & Hollomon, 2007). Functionally, all azoles are in the same cross-resistance group (FRAC, 2012), signifying the high risk of *Z. tritici* becoming cross-resistant to all DMIs. However, within the group it is recognised that there is much variation between the fungicides (Bean, 2008), and it is a very diverse group in terms of mobility *in planta*, disease control and intrinsic activity (Kendall *et al.,* 1994, Du Rieu & Burke, 1994). Hence, while cross-resistance between some azoles has been observed (Hermann & Gisi, 1994, Kildea *et al*., 2006), it is not always the case (Cools *et al*., 2005a, Fraaije *et al*., 2007, Kildea, 2009). Point mutations in the *CYP51* gene has been the major mechanism involved in reducing azole sensitivity (Bean, 2005, Cools *et al*., 2005a,

Cools *et al*., 2005b, Leroux *et al*., 2007, Stammler *et al*., 2008). In addition, over-expression of the target gene (Cools *et al*., 2012), possibly over-expression of the drug efflux transporter genes (Leroux & Walker, 2011, Fillinger *et al*., 2014) and combinations of all these mechanisms contribute to the slow but steady decline in azole efficacy. While the beginning of the decline in *in-vitro* sensitivity was seen in the early 1990's (Leroux *et al*., 2007), it was thought that azole insensitivity reached a plateau by 2008 (Stammler *et al*., 2008). However, recent analysis of epoxiconazole sensitivity results (Buitrago *et al*., 2014, Kildea & Glynn, 2014) showed a continuation of the decrease in sensitivity from 2010 to 2013. This is described in more detail in the introduction to Chapter 2.

1.4.3 The development and spread of fungicide resistance in *Zymoseptoria tritici*

Pathogen evolution occurs by changes in allele frequencies in populations (McDonald, 2004), and is driven by evolutionary processes such as mutation, migration, genetic drift, and mating system (Zhan & McDonald, 2004). Due to its mixed reproductive system and polycyclic lifecycle, *Z. tritici* is said to have high evolutionary potential (Zhan & McDonald, 2004). Frequent asexual reproduction creates a large effective population size which is adapted to its environment (Linde *et al*., 2002, Zhan & McDonald, 2004). This is an important feature of *Z. tritici*; as each individual propagule has a chance of acquiring a mutation (Anderson, 2005), larger populations will produce more mutations. In a strictly clonal pathogen, successive random mutational events would have to occur in a single variant before multiple resistance alleles would be found in combination. But, sexual reproduction in *Z. tritici* facilitates the rapid combining of resistance alleles (Brunner *et al*., 2008) which can be 'tested' in new environments, as the progeny are wind dispersed.

This introduction of genetic variation/novel alleles into the population by mutation and invasion is the first step in the process of evolution. While most new alleles do not survive, some do by random chance. If the new allele is fit enough to survive in a hostile

environment such as post-fungicide application, it will be able to reproduce in that environment. At this point, processes such as selection and gene-flow change the allele frequencies in the population (McDonald, 2004). This is known as the emergence phase of fungicide resistance, the first of three phases in the evolution of fungicide resistance; emergence, selection and adjustment (Van den Bosch *et al*., 2011). The emergence phase lasts from the introduction of the new fungicide until the resistant strain increases in number to a size where it is unlikely to die out by chance (Hobbelen *et al*., 2014). Following this, the increase of resistant strains as a proportion of the pathogen population can be described as the selection phase. Unlike the emergence phase, selection is not about the absolute number of resistant strains in the population but more about the rate of increase of the resistant population relative to the sensitive population (Van den Bosch *et al*., 2011). The use of fungicides in the presence of resistant strains undoubtedly increases the selective advantage of such strains and hence their proportion in a population. When the resistant subpopulation becomes so large that the field efficacy of the respective fungicide(s) is compromised and adjustment of disease management practices is necessary, this is known as the adjustment phase. Management of resistance during each phase may differ, but this thesis generally focusses on the selection phase.

1.5 Resistance management

The aim of fungicide anti-resistance strategies is to reduce the rate at which field resistance develops, at the same time as maintaining control of the disease, thereby increasing the 'effective life' of the product (van den Berg *et al.*, 2013). This is achieved by minimising the selection coefficient, i.e. the difference in fitness between the resistant and sensitive strains (van den Bosch *et al*., 2014) by either reducing the rates of increase of both sensitive and resistant strains, reducing the rate of increase of resistant strains relative to that of sensitive strains, or by reducing the exposure time of the target pathogen to the fungicide. Anti-

resistance recommendations, also known as resistance management tactics, are based on the overall premise that reducing the time that the pathogen is exposed to the fungicide would increase the effective life of that fungicide (van den Bosch *et al*., 2014). Recommendations include mixing or alternating different modes-of-action, limiting the number of applications of an individual fungicide, and choice of application dose (van den Bosch *et al*., 2014). In addition, reducing the levels of disease in the crop by utilising existing host resistance and suitable agronomic practices would ease the pressure on fungicides. Different versions of these tactics are used and previous studies on this subject have been reviewed recently by van den Bosch *et al*. (2014) who discuss nine separate tactics used to potentially minimise the selection coefficient. There are of course many complicating factors in resistance management, e.g. not only can populations be at different stages of resistance evolution to the different fungicides being used at the same time, where management tactics may vary depending on the stage (van den Bosch *et al*., 2014b), but also, in cases of polygenic resistance, individual isolates within a population can have different levels of resistance, potentially all levels from very sensitive to very resistant. In theory, this means that each fungicide resistance case should be managed on an individual basis. However this is not always practical and inferences from other studies may be the only information available for a particular pathosystem. So, even though there is much available information on fungicide resistance in *Z. tritici*, it is not complete.

Fungicides are conventionally mixed to extend the spectrum of activity and to improve the disease control seen with weaker products, but also as an insurance against resistance to one component and as a general anti-resistance tactic (van den Bosch *et al*., 2014b). From an anti-resistance point of view, most strains resistant to one component of a mixture are likely to be sensitive to the other component, and vice versa, (assuming cross-resistant fungicides are not mixed). Reviews by van den Bosch *et al*. (2014) and van den Bosch *et al*. (2014b)

highlighted that much empirical (Sanders *et al*., 1985, Samoucha & Gisi, 1987, Pijls & Shaw, 1997, Koller & Wilcox, 1999, Hollomon *et al*., 2002, Mavroidis & Shaw, 2002, Kuck & Mehl, 2004, Genet *et al*., 2006, Mavroeidi & Shaw, 2006, Thygesen *et al*., 2009, Perron *et al*., 2012) and modelling (Kable & Jeffery, 1980, Skylakakis, 1981, Levy *et al*., 1983, Kosman & Cohen, 1996, Birch & Shaw, 1997, Paveley *et al*., 2003, Shaw, 2007, Hobbelen *et al*., 2011b, Hobbelen *et al*., 2013, Mikaberidze *et al*., 2014) work has been done on the effects of mixtures on selection for resistance. Most of these publications consider mixtures with different modes–of-action, as the manufacturers recommend, and after a review of the literature van den Bosch *et al*. (2014) conclude that adding a mixing partner (low-risk) reduces selection for resistance to the high-risk component. However, some of the most popular and effective anti-fungal products for STB control in Ireland are azole mixtures which combine epoxiconazole and metconazole, prothioconazole and tebuconazole, and difenconazole and tebuconazole. The differential selection (where one fungicide is seen to select for a specific strain, and a different fungicide selects against the same strain) observed in azoles (Fraaije *et al*., 2007, Leroux *et al*., 2007) suggests that combinations of active ingredients from this group may be used without the expected detrimental effects of over-using fungicides with the same mode-of-action. Even so, these are mixtures of fungicides which have a similar risk of resistance development, and on which little research has been carried out (van den Bosch *et al*., 2014b).

Compared to the body of work on mixtures, less has been done on the effects of alternations (sequential application of fungicides) on the selection for resistant *Z. tritici* strains, and the research has been more theoretical (Kable & Jeffery, 1980, Birch & Shaw, 1997, Hobbelen *et al*., 2013) than empirical (Sanders *et al*., 1985, Bolton & Smith, 1988). The review by van den Bosch *et al*. (2014) discussed two possible scenarios for alternating fungicides. First, maintaining the basic fungicide program which uses an effective (and

probably high-risk of resistance) fungicide, but with the addition of a different mode-of-action between those applications. They conclude that this tactic did not alter selection, probably because the number of applications of the high-risk fungicide was not reduced. The second alternation scenario involves the replacement of one application of the high-risk fungicides with a different mode-of-action, i.e. reducing the number of applications of an individual a.i. and so reducing exposure time to that fungicide, which in turn reduces selection (van den Bosch *et al*., 2014). Similar to mixtures, when alternations have been studied, fungicides with different modes-of-action were mostly considered (van den Bosch *et al*., 2014). In the theoretical work that compares mixtures to alternations, it is clear that the difference between the two can depend on many factors, such as: initial frequency of resistant strains or the presence of fitness costs in resistant strains (Hobbelen *et al.,* 2013); spray coverage (Kable & Jeffery, 1980); dose used and whether two pesticides are suitable for mixing (Birch & Shaw, 1997). Even so, van den Bosch *et al*. (2014) conclude that mixtures are often the best strategy, but decisions should be made on a case-by-case basis.

Manufacturers' recommended dose rates presumably aim to keep the pathogen population to a minimum, which should decrease the chances of resistant mutations occurring (Zhan & McDonald, 2004). Conversely, large populations make it difficult for a mutant to build up to any damaging level, in the presence of so many sensitive types (van den Berg *et al.,* 2013). An alternative theory, reviewed by Shaw (2009), is that if no fungicide is applied there can be no selection, so if less fungicide is present, there will be less selection. When considering fungicide dose as an anti-resistance tactic, there are multiple options (van den Bosch *et al*., 2014): (i) Using split applications: where the same overall amount of fungicide is applied but over an increased number of applications; using this tactic, exposure time is increased and hence, selection increased; (ii) Manipulating the dose of mixture components: in this scenario, the dose of the high-risk fungicide is reduced relative to the dose of the low-

risk fungicide. This reduces the rate of increase of both resistant and sensitive strains, and so reduces the selection coefficient (van den Bosch *et al*., 2014). However, when two high-risk fungicides are mixed, both of which select for resistant strains, this tactic may not have the same effect and is likely to lead to stronger selection for the fungicide kept at the full dose (Shaw, 1993); (iii) Reduce the overall amount of fungicide but apply it over the same number of application times: this decreases the fitness difference between the resistant and sensitive strains, which in turn reduces selection (van den Bosch *et al*., 2014). This tactic goes against the general manufacturers' recommendation to only use at recommended dose rate. Considerable experimental, theoretical and review studies have been carried out on the subject of dose (Sanders *et al*., 1985, Shaw, 1989, Zziwa & Burnett, 1994, Burnett & Zziwa, 1997, Metcalfe & Shaw, 1998, Koller & Wilcox, 1999, Metcalfe *et al*., 2000, Shaw, 2000, Mavroidis & Shaw, 2002, Genet *et al*., 2006, Mavroeidi & Shaw, 2006, Shaw, 2007, Shaw, 2009, Gressel, 2010, Van den Bosch *et al*., 2011, Van den Berg *et al*., 2013, van den Bosch *et al*., 2014), many of which suggest that reducing rates can be an effective anti-resistance tactic. However, Van den Bosch *et al*. (2011) hypothesise that in the case of step-wise resistance development, it may be feasible that high doses reduce selection, but in practice it is unlikely and currently there is no experimental evidence to support this theory (van den Bosch *et al*., 2014).

1.6 Aims and objectives of this Ph.D.

Winter wheat yields are vulnerable to economically important scale of losses due to diseases. In general, crop losses can be reduced by adopting an integrated approach to crop management, including good cultivation practices, utilising available host resistance and where necessary, using available chemical control methods. For growers of winter wheat in Ireland and the other wheat growing regions in northern Europe, after tillage and nutrient management, controlling STB is often the main focus of crop management. While control of

STB is dependent upon chemical means, unfortunately fungicides are not the panacea they once were expected to be and loss of available active ingredients, either through resistance or regulation, is a real threat to control and crop losses. A reduction in field efficacy of azoles has already been seen. Further reductions in efficacy or direct removal of azoles from the market would expose the SDHIs to an increased risk of resistance development (Jess *et al*., 2014), which could eventually lead to the loss of all available fungicides for STB control. Hence, protection of these active ingredients now is of paramount importance to future winter wheat production in Ireland and Europe.

The overall aim of this project was to examine the effects of combining fungicides on the selection for resistance in *Z. tritici* populations, which in turn will contribute to future decision making processes regarding control of STB. In order to reach this goal, the following three chapters worked on accomplishing specific objectives:

Chapter 2: To clarify how resistance management tactics, in this case mixtures, alternations and reduced recommended dose rates of azoles, affect the selection for resistant phenotypes for each fungicide component, in addition to their effect on disease control and yield.

Chapter 3: To elucidate the target gene changes brought about by the application of azoles and combinations of azoles. In addition, an evolutionary history of the azole treated population was proposed, and can be used to predict what will happen with populations in the future.

Chapter 4: To clarify how resistance management tactics, in this case mixtures and reduced recommended dose rates of two different modes-of-action, an azole and an SDHI, affect the selection for resistant phenotypes for each fungicide component, as well as their effect on disease control and yield. In addition, this chapter also studied the baseline sensitivity of new SDHI active ingredients.

Chapter 2: Effect of azole fungicide mixtures, alternations and reduced dose rates on azole sensitivity in the wheat pathogen *Zymoseptoria tritici*

2.1 Introduction

Control of STB is currently largely reliant on the timely application of fungicides. Unfortunately the development and widespread occurrence of resistance to the quinone outside inhibitor (QoI) and methyl benzimidazole carbamate (MBC) classes of fungicides in European *Z. tritici* populations has reduced the number of effective groups of fungicides available for STB control (Fraaije *et al*., 2005) to the multi-sites, the DMIs and the SDHIs. The development of insensitivity in *Z. tritici* to these remaining chemistries poses a threat to the future control of STB in Europe.

Since their introduction, prior to the introduction of the QoIs and subsequent to the development of resistance to the QoIs, the azoles (the largest group within the DMIs) have been the backbone of STB control in winter wheat (Fraaije *et al*., 2007). All azoles have the same mode-of-action: they target the cytochrome P450 enzyme, specifically eburicol 14α-demethylase (coded for by *CYP51*) (Yoshida & Aoyama, 1987), and work by preventing the biosynthesis of ergosterol, which is required for functioning fungal cell membranes (Bean *et al*., 2009). For more than a decade, a progressive reduction in sensitivity to azoles has been observed in European *Z. tritici* populations (Stammler & Semar, 2011). This reduction in sensitivity has been attributed to a number of different mechanisms including amino-acid alterations in the target site (14α-demethylase or *CYP51*), overexpression of the target site, and perhaps, increased efflux of the fungicides (Cools & Fraaije, 2013). Since the early 1990s, alterations in the *CYP51* gene have been identified, many of which had only slight effects on sensitivity to the majority of azoles (Cools & Fraaije, 2013). However, these early alterations may have, over the past 10-15 years, facilitated the emergence of alterations which affect the binding of specific azoles, leading to a reduction in sensitivity (Mullins *et al*.,

2011). Many of these changes can alter the sensitivity to specific azoles differently, as highlighted by Fraaije *et al*. (2007). For example, the now common I381V mutation is strongly selected by both tebuconazole and metconazole but the same mutation is selected against by the imidazole, prochloraz. The mutation V136A, however, makes *Z. tritici* more sensitive to tebuconazole but less sensitive to prochloraz (Fraaije *et al*., 2007). Since 2008, strains of *Z. tritici* with reduced sensitivity to epoxiconzole and prothioconazole have become common in Ireland, but as these strains have predominantly had the *CYP51* alterations V136A and S524T (Stammler & Semar, 2011) they have maintained their sensitivity to metconazole and tebuconazole (O'Sullivan & Kildea, 2010). This apparent lack of complete cross-resistance suggests that using multiple azoles in combination, either as mixtures or sequentially, may provide a means of reducing selection for less sensitive strains while maintaining disease control (Cools & Fraaije, 2013).

Using combinations of fungicides with the intention of slowing down the selection for resistance usually include fungicides with different modes-of-action. However, due to the commercial preference for fungicide products with activity against multiple fungal targets, combining azoles has become increasingly common in fungicide programmes on winter wheat. Unfortunately, not much is known about how such combinations alter the evolution of *Z. tritici* sensitivity. Most of the few sources of empirical data available for azole mixtures measured only STB control (Kendall & Hollomon, 1994, Kendall *et al*., 1996, Du Rieu *et al*., 1994), rather than the impact on *Z. tritici* sensitivity. A single report included azole mixtures (imidazole and triazole fungicides) in the context of resistance management (Fraaije *et al*., 2011). It suggested that using combinations of azoles which differentially select specific *CYP51* alterations can lead to a reduction of mutations, but it depends on the components of the combination. Similarly, there is very little empirical information available on how alternations of azoles affect selection for reduced sensitivity. Hobbelen *et al*. (2013) reviewed

models which study the effects of mixtures and alternations as anti-resistance strategies and found that most were designed to study combinations of low- and high-risk fungicides. None of these models discussed in depth the mixing or alternation of fungicides which target the same site.

In addition to mixing and alternating fungicides, the reduction of fungicide dose has been suggested as an anti-resistance strategy (Birch & Shaw, 1997, Burnett & Zziwa, 1997, Genet *et al*., 2006, Mavroeidi & Shaw, 2005, Mavroeidi & Shaw, 2006, Metcalfe *et al*., 2000, Pijls & Shaw, 1997, Shaw, 2000, Shaw, 2007, Shaw, 2009, Van den Bosch *et al*., 2011, Zziwa & Burnett, 1994), particularly in the selection phase. However, where strains with reduced sensitivity are present in a large proportion of the population, reducing the recommended dose per application is likely to lead to a reduction in disease control, potentially making such a strategy impractical (Hobbelen *et al*., 2011b).

The aim of the work reported here was to test the following hypotheses. Firstly, that combinations of azoles, either in mixtures or alternated at different application timings, will slow the rate at which strains with reduced sensitivity to either fungicide is selected in field populations of *Z. tritici*. Secondly, that reduced doses at each application reduce selection for less sensitive strains. To test these hypotheses, field trials using commercially available products, in high disease pressure environments were combined with sensitivity testing of *Z. tritici* isolates sampled pre- and post-fungicide application. The products used are widely employed in Ireland, Europe and elsewhere, and are of great commercial relevance.

2.2 Materials and methods

Field trials were conducted during 2010-11 and 2011-12 at six locations throughout Ireland (Table 2.1). Experimental plots were situated in commercial fields, and aside from fungicide treatments all experimental plots were treated as the rest of the commercial crop. All trials

were laid out as complete randomised block designs with four replicate blocks, each containing 10 fungicide treatments and an un-treated control. Plots were 2.5m × 10m with a 30-40cm path between plots. *Zymoseptoria tritici* was allowed to develop naturally in each trial. Experimental treatments consisted of two foliar fungicide applications (referred to as T1 at GS 32-37 and T2 at GS 39-53 depending on location (Zadoks *et al*., 1974) of the triazoles epoxiconazole (Opus®, BASF) and metconazole (Caramba®, BASF) as solo products, in alternation with one another at the different timings or as a mixture of both (Gleam®, BASF), and all of the above at full and half the recommended dose (see Table 2.2 for further details). All fungicides were applied in 200 L/ha water using a knapsack sprayer with compressed air.

2.2.1 Disease and yield assessments

Disease was assessed at GS 69-73 on the flag leaf of ten main tillers chosen at random, approximately equidistant apart in each plot. The percentage leaf area with STB was visually estimated. Plots were harvested each year using a specially adapted combine harvester. The grain from each plot was weighed and the moisture content determined in a representative sample from each plot. Yields were then calculated as t/ha at 15% moisture.

2.2.2 Sampling *Zymoseptoria tritici*

To determine the distribution of fungicide sensitivity in the *Z. tritici* population prior to spraying, each location was sampled. In 2011 approximately 100 diseased leaves and in 2012 approximately 50 diseased leaves were collected from each of the trial site locations, sampled uniformly from across the whole site. At the second sampling time (six weeks post T2 fungicide application), approximately 40 diseased flag leaves were collected, without regard to actual amount of disease, at roughly equal distances apart within each plot and avoiding ends and edges of plots. At the Stamullen and Knockbeg locations in 2011, disease levels were too low six weeks after T2 so sampling was conducted eight weeks after the T2 fungicide application. At these two locations, disease levels were still low after eight weeks

and diseased leaves were actively sought. The diseased leaves from each plot were air dried for five days at room temperature and then stored at -20 °C awaiting pathogen isolation.

2.2.3 Isolating *Zymoseptoria tritici*

Isolations were carried out according to Kildea (2009). Briefly, diseased leaves (cut to fit four in a ten cm petri dish) were washed in running tap water for two hours before being surface sterilised (immersed in 70% ethanol for 20 seconds, 10% sodium hypochlorite for two minutes and triple rinsed with sterile distilled water). The leaves were subsequently dried using tissue paper and placed, exposed pycnidia facing upwards, on water agar, then incubated in the dark at 18 °C for 24-48 hours to promote sporulation. Following incubation, a single cirrus from each leaf was picked using a fine sterile needle and streaked onto potato glucose agar (PGA) (Sigma-Aldrich, St. Louis, MO, USA) amended with 50 mg L^{-1} chloramphenicol and 50 mg L^{-1} streptomycin. Petri dishes were sealed and incubated in the dark at 18 °C for 4-6 days. Isolates were sub-cultured onto antibiotic amended PGA (as above), sealed and incubated at 18° for a further three days. Pure cultures were scraped from the plates and individually stored in 30% glycerol at -80 °C until further use.

Table 2.1 Location details: year each location was included, timing of fungicide applications and growth stage at which fungicides were applied (GS)

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

[a] Resistant rating on a scale of 1-9, 1 = susceptible, 9 = resistant (DAFM https://www.agriculture.gov.ie/publications/2013/)
[b] GS Growth stage (Zadoks *et al*., 1974)

Table 2.2 Treatments used: application pattern, dose rates applied, fungicides used and actual amount of active ingredient (a.i.) at each treatment time

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

[a] Abbreviations denote the first and second sprays. Un-T= un-treated control; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose
[b] Application dose at Treatment 1 and Treatment 2; 1 = the full label recommended dose, 0.5 = half the label recommended dose
[c] Epoxiconazole = Opus Max, Metconazole = Caramba, Epoxiconazole + Metconazole = Gleam. All fungicides are BASF products
[d] Active ingredient (a.i.) per litre of product; Opus max: 83 g/l; Caramba: 60 g/l; Gleam: 37.5 g/l epoxiconazole + 27.5 g/l metconazole

2.2.4 *In vitro* sensitivity testing

The sensitivity of all isolates to epoxiconazole and metconazole was determined using a microtitre plate assay as described by Kildea (2009). Technical grade epoxiconazole and metconazole (purchased from Sigma-Aldrich) were dissolved in 100% methanol and added to Potato Dextrose Broth (PDB) (Sigma-Aldrich Co.) to give final test concentrations of 30, 10, 3.3, 1.1, 0.37, 0.123, 0.04, and 0 mg $L^{-1}$of which 150 µl were added to wells of flat bottomed sterile 96-well microtitre plates (Sarsted AG & Co., Germany). Inoculum of each isolate was produced by spotting 30 µl of the pure culture stock solutions described in 2.2.3 on PGA and incubated for three days at 18 °C. Test suspensions were made in PDB and adjusted to a final concentration of $1x10^5$ spores/ml, of which 50 µl was added to the wells of the microplates containing the different fungicide concentrations. Each plate consisted of a negative control (PDB only), a positive control (isolate 4465, of Irish origin and kindly supplied by BASF) and 10 experimental isolates. In some exceptional cases, isolate 4465 did not produce sufficient spores to allow for the inclusion of a positive control in all test plates. All plates were tested in replicate at the same time, sealed with parafilm, stored in sealable bags to reduce condensation and incubated in the dark at 18 °C for 7 days. Due to the large number of isolates in the whole experiment, isolates from the same plot, replicate, location or treatment were not necessarily tested on the same date. Fungal growth was assessed as a measure of light absorbance at 405 nm using Synergy-HT plate reader and Gen5™ microplate software (BioTek Instruments, Inc., USA).

2.2.5 Data analysis

The fungicide dose reducing growth in the microplate wells by 50% (EC_{50}), estimated by optical density, was determined by fitting a logistic curve to percentage inhibition data generated from the optical density measurements for each isolate using the computer program XLfit (IDBS Inc., UK). Where a plate had a reference isolate, EC_{50} values from that plate

were adjusted for differences in the reference isolate between plates, according to Mavroeidi and Shaw (2005). The subsequent analysis was weighted to allow for the increased variance of observations from plates where the EC_{50} of the reference isolate could not be measured. Observations from plates with a successful reference isolate measurement were given a weight of 1 and a value of 1-(variance within the standards/variance in isolates from plates with standards) given otherwise. All statistical analyses were carried out in GenStat 14th Edition (VSN International Ltd. United Kingdom). Differences between plate replicates were analysed using ANOVA.

As the numbers of isolates with successfully measured EC_{50} values varied between plots, the data were not balanced. Differences in EC_{50} values between treatments were therefore analysed and means constructed using Restricted Maximum Likelihood (REML). Data from the early sampling time (Pre-T) were analysed using REML, whilst data from the later sampling time were analysed using REML with contrasts (Crawley, 2005), using the FCONTRASTS procedure. In the model, treatment (11 levels) was considered a fixed effect, whilst location (six levels) and rep (four levels) and location.treatment were considered random effects. Contrasts were estimated separately for epoxiconazole and metconazole sensitivity. Principal Component Analysis (PCA) was used to determine the common effects of using epoxiconazole and metconazole on overall sensitivity, and to look at how selection by epoxiconazole and metconazole affected specific resistance to each fungicide. Sensitivity data were subjected to PCA based on sums of squares and products. Principal component scores, PC1 and PC2, were analysed using REML with contrasts.

Disease severity data were square root (sqrt) transformed and differences between treatments were analysed using ANOVA with a factorial plus control procedure. Disease severity data were correlated with the sensitivity data using general linear regression including differences in sensitivity between locations as a factor. Differences in yield were

analysed using ANOVA with a factorial plus control procedure and the relationship between yield and disease control was estimated using general linear regression including location differences.

2.3 Results

The sensitivity of 3703 single pycnidial *Z. tritici* isolates were determined. Of these, all were tested for sensitivity to epoxiconazole, but due to contamination in some plates, only 3683 isolates were tested for sensitivity to metconazole. Sensitivity data were not determined for the half-dose alternation treatments in 2011-12. There was no statistical difference ($P = 0.9$) between replicate plate measurements of each isolate and therefore mean EC_{50} values for each isolate were used in the subsequent analysis. The mean sensitivity of *Z. tritici* isolates to epoxiconazole and metconazole varied with treatment (Table 2.3a & Table 2.3b).

2.3.1 Variability before fungicide applications

Isolates from the population prior to fungicide applications (Pre-T) ranged in sensitivity to epoxiconazole from a $\log_{10}EC_{50}$ (mg L^{-1}) of -2.38 to 0.51 (a variation factor of 776), and to metconazole from a $\log_{10}EC_{50}$ (mg L^{-1}) of -2.38 to 1.35 (a variation factor of 5370) (Figure 2.1). At this sampling time epoxiconazole sensitivity was similar at all locations (Figure 2.1, $P = 0.15$), but metconazole sensitivity differed between locations (Figure 2.1, $P < 0.001$).

Table 2.3 Mean sensitivity ($log_{10}EC_{50}$ mg L^{-1}) of isolates from individual treatments, including pre-treatment, over all locations to (a) epoxiconazole and (b) metconazole, and broken down into treatment means per location

a	Epoxiconazole sensitivity ($log_{10}EC_{50}$ mg L^{-1})								
	Experiment average			Individual location					
Treatment[a]	*n*	Mean	*SE*	Duleek (*n* = 770)	Julienstown (*n* = 449)	Killeagh (*n* = 502)	Knockbeg (*n* = 710)	Oak Park (*n* = 490)	Stamullen (*n* =7 82)
Pre-T	176	-0.479	0.0711	-0.457	-0.427	-0.480	-0.641	-0.449	-0.403
Un-T	357	-0.377	0.0687	-0.438	-0.355	-0.344	-0.464	-0.330	-0.328
EE	391	-0.042	0.0685	-0.112	0.141	-0.012	-0.135	-0.114	-0.014
MM	388	-0.209	0.0686	-0.151	0.001	-0.292	-0.393	-0.347	-0.079
ee	325	-0.131	0.0691	-0.258	-0.055	0.031	-0.369	-0.218	0.079
mm	356	-0.292	0.0688	-0.274	-0.199	-0.283	-0.536	-0.269	-0.195
EM	379	-0.173	0.0687	-0.235	0.077	-0.368	-0.258	-0.266	0.006
ME	371	-0.054	0.0687	-0.058	0.140	0.038	-0.313	-0.302	0.162
em	168	-0.167	0.0832	-0.191	*	*	-0.404	*	0.030
me	172	-0.111	0.0825	-0.011	*	*	-0.435	*	0.039
EMEM	313	-0.034	0.0694	0.081	0.370	-0.189	-0.342	-0.133	-0.012
emem	307	-0.044	0.0692	0.142	0.217	-0.181	-0.321	-0.054	-0.090

Table 2.3 contd.

b	Metconazole sensitivity ($log_{10}EC_{50}$ mg L^{-1})								
	Experiment average			Individual location					
Treatment[a]	*n*	Mean	*SE*	Duleek (*n* = 762)	Julienstown (*n* = 448)	Killeagh (*n* = 503)	Knockbeg (*n* = 708)	Oak Park (*n* = 489)	Stamullen (*n* = 773)
Pre-T	176	-0.780	0.0606	-0.893	-0.713	-0.505	-0.957	-0.865	-0.728
Un-T	350	-0.765	0.0583	-0.902	-0.918	-0.837	-0.730	-0.525	-0.678
EE	389	-0.650	0.0581	-0.694	-0.725	-0.672	-0.717	-0.653	-0.449
MM	388	-0.507	0.0582	-0.635	-0.502	-0.568	-0.540	-0.509	-0.293
ee	325	-0.673	0.0586	-0.856	-0.864	-0.679	-0.733	-0.508	-0.406
mm	356	-0.533	0.0583	-0.631	-0.679	-0.385	-0.649	-0.423	-0.437
EM	379	-0.507	0.0582	-0.690	-0.357	-0.595	-0.615	-0.368	-0.406
ME	366	-0.591	0.0584	-0.654	-0.514	-0.565	-0.666	-0.706	-0.444
em	166	-0.641	0.0766	-0.823	*	*	-0.821	*	-0.289
me	170	-0.570	0.0758	-0.609	*	*	-0.734	*	-0.403
EMEM	312	-0.453	0.059	-0.419	-0.187	-0.508	-0.619	-0.614	-0.378
emem	306	-0.505	0.0587	-0.499	-0.390	-0.525	-0.642	-0.529	-0.452

[a] Treatment information in Table 2.2. Briefly, Pre-T=pre-treatment sample, Un-T= un-treated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose

n = number of isolates per group

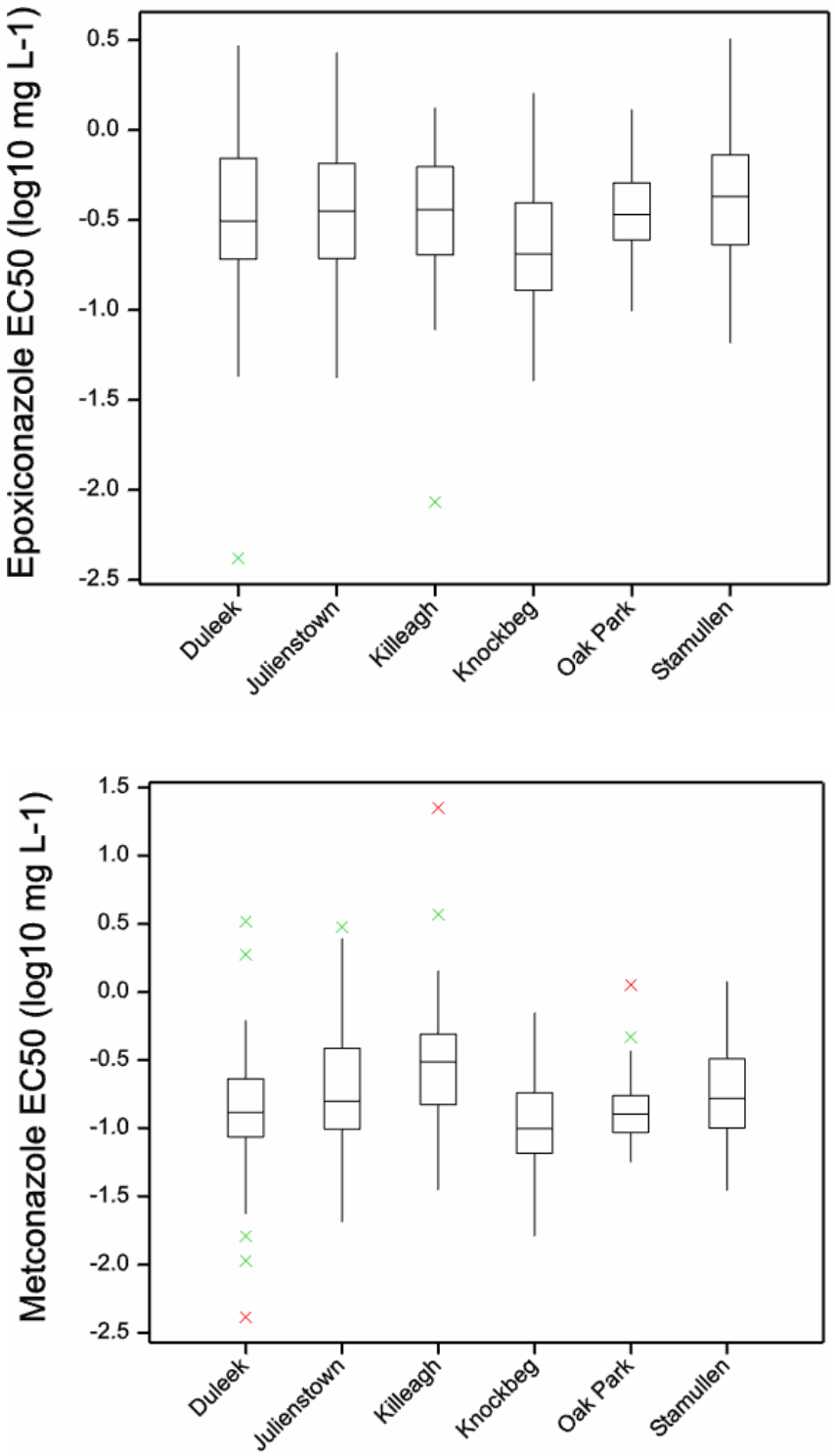


Figure 2.1 Frequency distribution of $log_{10}EC_{50}$ values for epoxiconazole sensitivity (top) and metconazole sensitivity (bottom) from Pre-T collections of *Zymoseptoria tritici* sampled from each of the six locations, illustrated with box and whisker plots. The line through the box represents the median. Number of Pre-T isolates tested from each location; Duleek n = 33; Julienstown n = 29; Killeagh n = 20; Knockbeg n = 25; Oak Park n = 21; Stamullen n = 48

2.3.2 Main contrasts

Specific differences between the effects of particular treatment patterns at individual locations and years of observation would not be relevant to the choice of overall resistance strategy, so main effect contrasts are reported, using location and location interactions as random factors in the mixed effect REML model. *Zymoseptoria tritici* treated in any way with either of the fungicides became less sensitive to both epoxiconazole and metconazole than *Z. tritici* from the un-treated plots ($P < 0.001$ and $P < 0.001$ respectively, Table 2.4a and Table 2.4b, contrast 1) with large reductions in sensitivity at some locations, for example at Duleek, Julienstown and Killeagh, there was a two to four-fold decrease in sensitivity to epoxiconazole but at Stamullen, a 44-fold decrease was observed (Table 2.3a). All treatments containing epoxiconazole saw a larger shift in sensitivity than those treatments without ($P < 0.001$, Table 2.4a, contrast 2). The same was seen for metconazole sensitivity, where all treatments containing metconazole measured a larger shift in sensitivity than treatments without metconazole ($P = 0.002$, Table 2.4b, contrast 2). There was no significant difference between the effect of the mixture and the solo epoxiconazole on epoxiconazole sensitivity ($P = 0.3$, Table 2.4a, contrast 3) or between the effect of the mixture and the solo metconazole on metconazole sensitivity ($P = 0.42$, Table 2.4b, contrast 3). *Zymoseptoria tritici* isolates from treatments which received two applications of epoxiconazole were less sensitive than those that received only one, although the difference was not quite significant ($P = 0.09$, Table 2.4a, contrast 4). For metconazole sensitivity, the treatments which applied metconazole twice caused a significant decrease in sensitivity ($P = 0.03$, Table 2.4b, contrast 4) compared to the treatments which applied metconazole only once. The order in which the a.i. was applied in the alternation had no effect on epoxiconazole sensitivity ($P = 0.1$, Table 2.4a, contrast 5) or metconazole sensitivity ($P = 0.9$, Table 2.4b, contrast 5).

Table 2.4 Independent single degree of freedom contrasts between treatments in (a) epoxiconazole and (b) metconazole sensitivity

a			Treatment coefficients* included in each contrast question										
Contrast	*Contrast sizes*	*P* [a]	Un-T [b]	EE	MM	ee	mm	EM	ME	em	me	EMEM	emem
1. Effect of fungicide	0.023	<0.001	-10	1	1	1	1	1	1	1	1	1	1
2. Treatments with any epoxiconazole cf. those without	0.031	<0.001	0	1	-4	1	-4	1	1	1	1	1	1
3. Mixture cf epoxiconazole solo	-0.024	0.3	0	1	0	1	0	0	0	0	0	-1	-1
4. Treatments with two applications of epoxiconazole cf. those with one	0.0322	0.09	0	1	0	1	0	-1	-1	-1	-1	1	1
5. Order of application of a.i. in alternation	-0.043	0.1	0	0	0	0	0	1	-1	1	-1	0	0
6. Effect of dose	0.023	0.2	0	1	1	-1	-1	1	1	-1	-1	1	-1
7. Dose interaction with contrast 2	-0.004	0.5	0	1	-4	-1	4	1	1	-1	-1	1	-1
8. Dose interaction with contrast 3	0.02	0.4	0	1	0	-1	0	0	0	0	0	-1	1
9. Dose interaction with contrast 4	0.006	0.8	0	1	0	-1	0	-1	-1	1	1	1	-1
10. Dose interaction with contrast 5	-0.16	0.6	0	0	0	0	0	1	-1	-1	1	0	0

Table 2.4 cont.

b			Treatment coefficients* included in each contrast question										
Contrast	*Contrast sizes*	*P* [a]	Un-T [b]	EE	MM	ee	mm	EM	ME	em	me	EMEM	emem
1. Effect of fungicide	0.018	<0.001	-10	1	1	1	1	1	1	1	1	1	1
2. Treatments with any metconazole cf. those without	0.025	0.002	0	-4	1	-4	1	1	1	1	1	1	1
3. Mixture cf metconazole solo	-0.019	0.4	0	0	1	0	1	0	0	0	0	-1	-1
4. Treatments with two applications of metconazole cf. those with one	0.042	0.03	0	0	1	0	1	-1	-1	-1	-1	1	1
5. Order of application of a.i. in alternation	0.003	0.9	0	0	0	0	0	1	-1	1	-1	0	0
6. Effect of dose	0.022	0.2	0	1	1	-1	-1	1	1	-1	-1	1	-1
7. Dose interaction with contrast 2	0.002	0.7	0	-4	1	4	-1	1	1	-1	-1	1	-1
9. Dose interaction with contrast 3	-0.008	0.7	0	0	1	0	-1	0	0	0	0	-1	1
8. Dose interaction with contrast 4	-0.007	0.7	0	0	1	0	-1	-1	-1	1	1	1	-1
10. Dose interaction with contrast 5	0.041	0.2	0	0	0	0	0	1	-1	-1	1	0	0

[a] P-value is based on the F-distribution
[b] Treatment information in Table 2.2. Briefly, Un-T= un-treated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose-
*Each coefficient denotes the weight by which a mean value was multiplied to calculate the contrast

Epoxiconazole sensitivity did not differ between locations (P = 0.15) whereas metconazole sensitivity did (P < 0.001). Even though a larger shift in sensitivity to both fungicides was observed after full doses, (ns, P = 0.12, Table 2.3) averaged over all treatments the difference between half doses and full doses was not significant for either epoxiconazole or metconazole sensitivity (P = 0.2 and P = 0.2 respectively, Table 2.4a & Table 2.4b, contrast 6). Interactions between dose and contrasts 2-5 were all non-significant (Table 2.4a & Table 2.4b, contrasts 7, 8, 9 and 10).

2.3.3 Principal components analysis

The first principal component (PC1: a measure of common sensitivity to both epoxiconazole and metconazole) accounted for 75% of the total variation amongst the isolates (Figure 2.2). The loadings for each variable were almost equal, meaning both epoxiconazole and metconazole sensitivity made an almost equal contribution to the variation between isolates. PC1 differed significantly between the un-treated and treated plots (P < 0.001, Table 2.5, contrast 1) and between the solo products and the mixture (P = 0.002, Table 2.5, contrast 3). No other contrasts were significant. The second principal component (PC2: a measure of the distinction between epoxiconazole and metconazole sensitivity) accounted for the remaining 25% of total variation (Figure 2.2). PC2 differed between the solo active ingredients (P < 0.001, Table 2.6, contrast 2). Also, the order of active ingredients in the alternation treatments affected selection on PC2 (P = 0.05, Table 2.6, contrast 5) but this effect differed between doses (P = 0.01, Table 2.6, contrast 10). All other contrasts were non-significant.

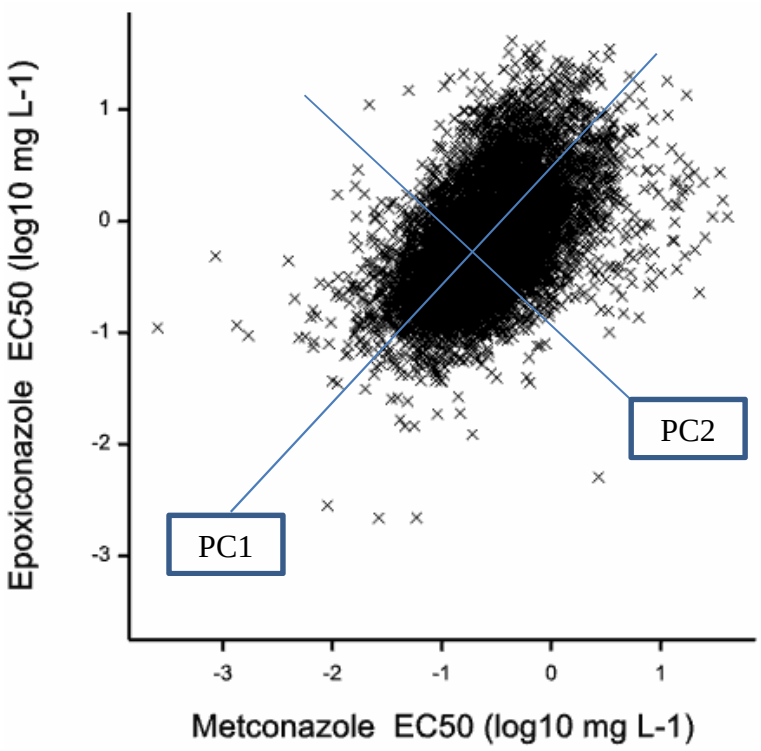


Figure 2.2 Correlation matrix with principal component axes superimposed. PC1 accounts for 75% variation, PC2 accounts for 25% variation

Table 2.5 Independent single degree of freedom contrasts between treatments with common azole sensitivity (PC1 in a principal component transformation of the data)

Contrast	*Contrast sizes*	*P* [a]	Treatment coefficients* included in each contrast question										
			Un-T [b]	EE	MM	ee	mm	EM	ME	em	me	EMEM	emem
1. Effect of fungicide	0.029	<0.001	-10	1	1	1	1	1	1	1	1	1	1
2. Epoxiconazole solo cf. metconazole solo	0.014	0.6	0	1	-1	1	-1	0	0	0	0	0	0
3. Mixture cf. solo fungicides	0.057	0.002	0	1	1	1	1	0	0	0	0	-2	-2
4. Treatments with two applications of an azole cf. those with one	0.006	0.8	0	1	1	1	1	-1.5	-1.5	-1.5	-1.5	1	1
5. Order of application of a.i. in alternation	0.035	0.3	0	0	0	0	0	1	-1	1	-1	0	0
6. Effect of dose	0.033	0.12	0	1	1	-1	-1	1	1	-1	-1	1	-1
7. Dose interaction with contrast 2	-0.003	0.9	0	-1	1	1	-1	0	0	0	0	0	0
8. Dose interaction with contrast 3	0.006	0.7	0	1	1	-1	-1	0	0	0	0	-2	2
9. Dose interaction with contrast 4	0.001	0.9	0	1	1	-1	-1	-1.5	-1.5	1.5	1.5	1	-1
10. Dose interaction with contrast 5	0.014	0.7	0	0	0	0	0	1	-1	-1	1	0	0

[a] P-value is based on the F-distribution

[b] Treatment information in Table 2.2. Briefly, Un-T= un-treated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose

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

Table 2.6 Independent single degree of freedom contrasts between treatments in the difference between epoxiconazole and metconazole sensitivity (PC2 in a principal component transformation of the data)

Contrast	*Contrast sizes*	*P* [a]	Treatment coefficients* included in each contrast question										
			Un-T [b]	EE	MM	ee	Mm	EM	ME	em	me	EMEM	emem
1. Effect of fungicide	0.0005	0.9	-10	1	1	1	1	1	1	1	1	1	1
2. Epoxiconazole solo cf. metconazole solo	0.107	<0.001	0	1	-1	1	-1	0	0	0	0	0	0
3. Mixture cf. solo fungicides	0.001	0.9	0	1	1	1	1	0	0	0	0	-2	-2
4. Treatments with two applications of an azole cf. those with one	-0.01	0.2	0	1	1	1	1	-1.5	-1.5	-1.5	-1.5	1	1
5. Order of application of a.i. in alternation	0.034	0.05	0	0	0	0	0	1	-1	1	-1	0	0
6. Effect of dose	-0.001	0.9	0	1	1	-1	-1	1	1	-1	-1	1	-1
7. Dose interaction with contrast 2	-0.0002	0.9	0	-1	1	1	-1	0	0	0	0	0	0
9. Dose interaction with contrast 3	0.012	0.1	0	1	1	-1	-1	0	0	0	0	-2	2
8. Dose interaction with contrast 4	0.01	0.2	0	1	1	-1	-1	-1.5	-1.5	1.5	1.5	1	-1
10. Dose interaction with contrast 5	-0.044	0.01	0	0	0	0	0	1	-1	-1	1	0	0

[a] P-value is based on the F-distribution

[b] Treatment information in Table 2.2. Briefly, Un-T= un-treated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose

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

2.3.4 Disease severity and its relationship with selection

Un-treated control plots had the most disease at all locations ($P < 0.001$); with an average of 12% (3.46 sqrt %) disease severity on the flag leaf at GS 69-73 (Figure 2.3).

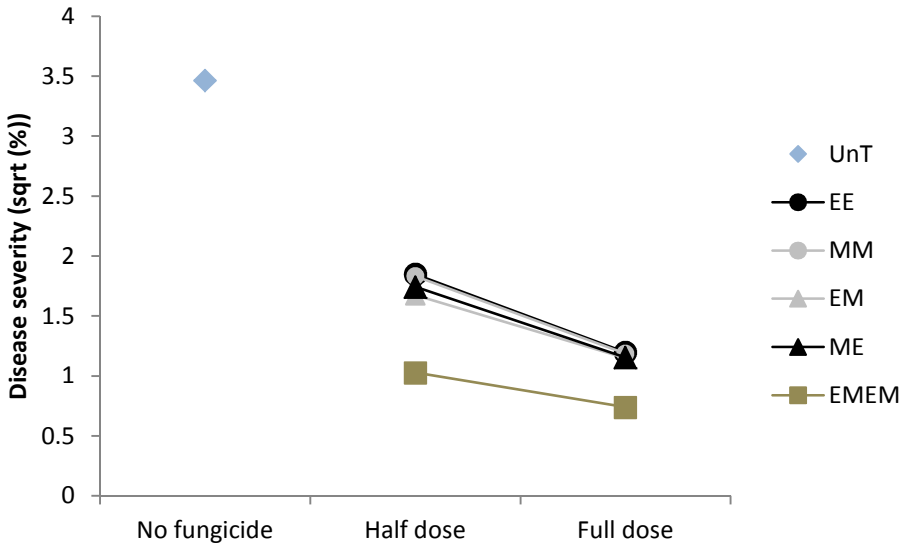


Figure 2.3 Effect of individual treatments on disease severity on the flag leaf at GS 69-73 averaged over all six locations. Disease severity refers to the proportion of the flag leaf covered in Septoria tritici blotch (square root transformed). Each line is used to connect the full and half doses of the same application pattern. Treatment information: abbreviations denote the first and second sprays. E: epoxiconazole; M: metconazole; UnT: un-treated control

Significant differences in disease severity in the un-treated plots were observed between locations ($P < 0.001$); Julienstown had the most disease in un-treated plots, with 25% (4.964 sqrt %) of the flag leaf infected with STB, and Stamullen had the least, with 0.3% (0.510 sqrt %). Significant differences in disease severity in the treated plots were observed between locations ($P < 0.001$, Table 2.7); Stamullen had the least disease after treatment, with 0.05% (0.22 sqrt %) and Julienstown and Killeagh had the most, both with 3.5% (1.87 sqrt %) disease on the flag leaf. The full dose treatments generally controlled STB better than their half dose counterparts, but the effect depended on locations ($P = 0.015$, Table 2.7). Disease control differed between treatments (mean of full and half doses of each product) ($P < 0.001$,

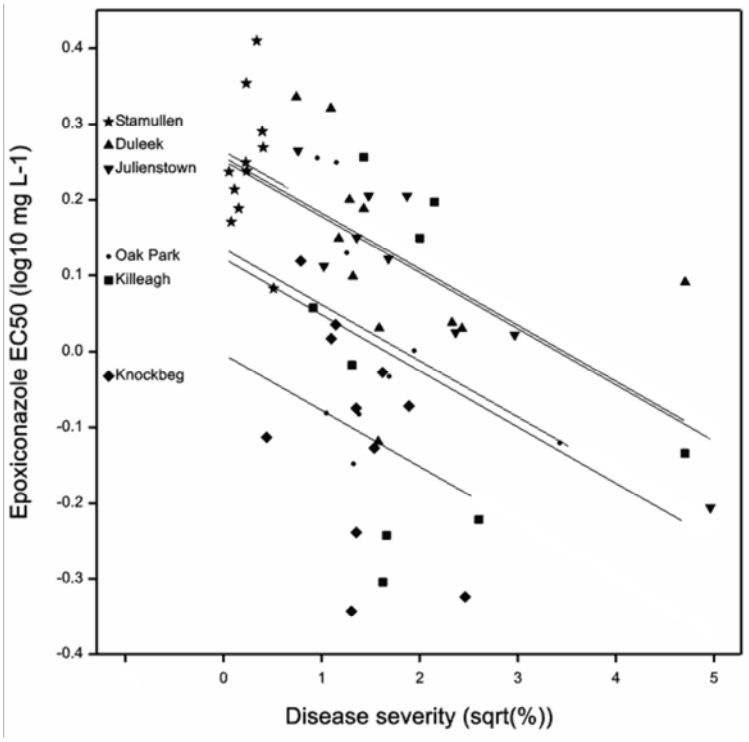
Table 2.7); with the mixture providing significantly better disease control (0.78% disease severity (0.88 sqrt %)) than any of the other treatments (average 2.17% (1.47 sqrt %) disease severity). There was an inverse relationship between disease severity and EC_{50} values of isolates to epoxiconazole and metconazole (Figure 2.4, $R^2 = 0.48$, $P < 0.001$ and Figure 2.4, $R^2 = 0.60$, $P < 0.001$ respectively; common slope but intercepts differing between locations).

Table 2.7 Disease severity at GS 69-73 (measured as average percent STB per surface area of the flag leaf, square root transformed) between treatments at each location. Underneath are the results of a cross-location analysis using factorial plus control procedure

	Location						Mean
Treatment[a]	Duleek	Julienstown	Killeagh	Knockbeg	Oak Park	Stamullen	
Un-T	4.71	4.96	4.71	2.46	3.43	0.51	3.46
EE	1.43	1.48	2.00	0.79	1.26	0.23	1.20
ee	2.43	2.97	2.15	1.62	1.69	0.23	1.85
MM	1.18	1.87	1.66	1.30	1.05	0.06	1.19
mm	2.33	2.37	2.60	1.35	1.94	0.40	1.83
EM	1.59	1.02	1.63	1.10	1.33	0.24	1.15
em	1.58	2.55	2.57	1.54	1.65	0.16	1.67
ME	0.74	1.68	1.43	1.35	1.38	0.34	1.15
me	1.32	2.60	2.32	1.89	1.92	0.40	1.74
EMEM	1.29	0.76	0.91	0.44	0.96	0.08	0.74
emem	1.09	1.36	1.31	1.14	1.15	0.11	1.03
Mean	1.79	2.15	2.12	1.36	1.61	0.25	1.55

Factorial plus control	*P*	*LSD (5% level)*
Location	< **0.001**	0.579
Product[b]	< **0.001**	0.276
Rate[c]	< **0.001**	0.247
Location.Product	0.11	0.676
Location.Rate	0.015	0.605
Product.Rate	0.5	0.319
Location.Product.Rate	0.47	0.781

[a] Treatment information in Table 2.2. Briefly, Un-T= un-treated control, abbreviations denote the first and second sprays; E or e: epoxiconazole, M or m: metconazole; uppercase: full dose; lowercase: half dose
[b] Product is full and half rates of each treatment compared; EE+ee, MM+mm, and EMEM+emem
[c] Full rates cf. half rates; EE+MM+EMEM cf. ee+mm+emem



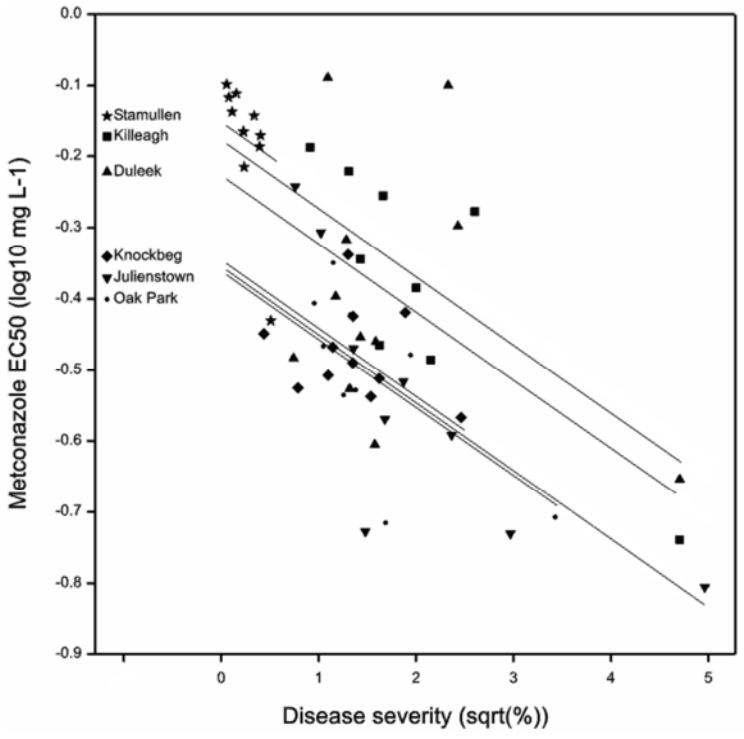


Figure 2.4 Fitted and observed relationship between (top) epoxiconazole sensitivity and disease severity, R^2 = 0.48, P < 0.001; common slope = -0.074; intercept for Duleek = 0.256; Julienstown = 0.252; ; Killeagh = 0.122; Knockbeg = -0.004; Oak Park = 0.135; Stamullen = 0.264 and (bottom) metconazole sensitivity and disease severity R^2 = 0.60, P < 0.001, common slope = -0.096; intercept for Duleek = -0.227; Julienstown = -0.355; Killeagh = -0.177; Knockbeg = -0.345; Oak Park = -0.361; Stamullen = -0.153

2.3.5 Effects if fungicides on yield

Un-treated control plots yielded significantly less than treated plots ($P < 0.001$, Table 2.8). Yield improvements after fungicide application varied between locations ($P < 0.001$, Table 2.8). Oak Park, Duleek and Knockbeg each had an improvement of 2 t/ha after fungicide treatments (mean of all treated plots) whereas Stamullen had the lowest with an improvement of only 0.1 t/ha, consistent with the low un-treated severity. Averaged over all treatments, full doses provided significantly higher yield than the half doses, and the half doses were significantly better than no fungicide ($P = 0.001$, Table 2.8). No differences in yield were seen between the two solo a.i.s, the two alternations or the mixture ($P = 0.17$, Table 2.8). There was a significant inverse relationship between disease and yield; but both the slope and intercept of this varied between locations (Figure 2.5, $R^2 = 0.98$, $P = 0.014$).

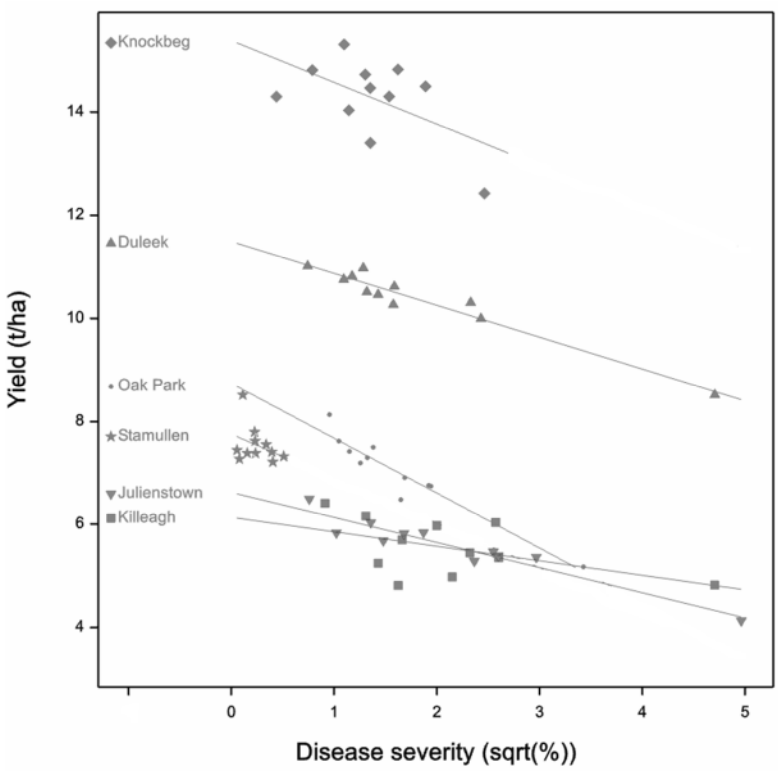


Figure 2.5 Fitted and observed relationship between yield and disease severity $R^2 = 0.98$, P = 0.014; Duleek: y= 11.49 + -0.-0.617 x; Julienstown: y = 6.62 + -0. 486x; Killeagh: y = 6.13 + -0.28x; Knockbeg: y = 15.4 + -0.814x; Oak Park: y = 8.74 + -1.069x; Stamullen: y = 7.75 + -0.862x

Table 2.8 Yield (t/ha) after treatments at each location, with a cross-location analysis using factorial plus control procedure

Treatment[a]	Location						Mean
	Duleek	Julienstown	Killeagh	Knockbeg	Oak Park	Stamullen	
Un-T	8.52	4.13	4.82	12.42	5.17	7.32	7.07
EE	10.46	5.68	5.97	14.82	7.19	7.8	8.65
ee	10	5.36	4.98	14.83	6.91	7.62	8.28
MM	10.82	5.84	5.7	14.73	7.62	7.44	8.69
mm	10.31	5.28	5.35	13.4	6.74	7.41	8.08
EM	10.62	5.83	4.82	15.32	7.3	7.38	8.54
em	10.27	5.47	6.03	14.3	6.48	7.38	8.32
ME	11.02	5.82	5.24	14.47	7.5	7.55	8.6
me	10.52	5.39	5.44	14.5	6.76	7.21	8.3
EMEM	10.98	6.49	6.41	14.3	8.14	7.27	8.93
emem	10.76	6.03	6.16	14.04	7.42	7.41	8.63
Mean	10.39	5.57	5.54	14.29	7.02	7.44	8.37

Factorial plus control	*P*	*LSD (5% level)*
Location	**<0.001**	0.891
Product[b]	0.17	0.425
Rate[c]	**0.001**	0.38
Location.Product	0.6	1.04
Location.Rate	0.5	0.93
Product.Rate	0.8	0.491
Location.Product.Rate	0.9	1.20

[a] Treatment information in Table 2.2. Briefly, Un-T= un-treated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose
[b] Full and half rates of each treatment (Product) compared; EE+ee, MM+mm, EM+em, ME+me and EMEM+emem
[c] Full rates cf. half rates; EE+MM+EM+ME+EMEM cf. ee+mm+em+me+emem

2.4 Discussion

To prolong the effective life of fungicides, strategies of use which delay both emergence of new resistant strains and selection for existing resistant strains, without compromising yield, are needed. In the experiments here, where STB was the dominant disease, yields achieved were directly related to the control of disease. Unfortunately, the success of the disease control was inversely related to the sensitivity of *Z. tritici* to those fungicides following treatment. This confirms the findings of Mavroeidi and Shaw (2006) who demonstrated that when the azole fluquinconazole was applied as a solo product, the greatest selection occurred

where the best control was achieved. In the current study, there was no evidence that the slope of this relationship differed between locations, thought the sensitivity of the population in un-treated plots did differ (Figure 2.4). Whilst the use of six different locations with varying sensitivity to both epoxiconazole and metconazole undoubtedly presents difficulties in determining the effects of individual fungicide treatments, the results presented are a realistic representation of the response of the Irish *Z. tritici* population, given the variation in azole sensitivities which exists.

With high levels of phenotypic variation between isolates at each location early in the season, a wide base from which selection could occur was present. Irrespective of application pattern (solo, mixture or alternation) or dose, all fungicide treatments significantly decreased the sensitivity of *Z. tritici* to both epoxiconazole and metconazole when compared to the un-treated controls. Results from the REML and PCA showed that each fungicide selected differentially for strains with reduced sensitivity. Even though epoxiconazole and metconazole target the same gene there is evidence which shows that different azoles select for different *CYP51* genotypes (Fraaije *et al*., 2007, O'Sullivan & Kildea, 2010, Stammler & Semar, 2011). Current populations of *Z. tritici* in Ireland contain a diversity of *CYP51* alleles and genotypes (Chapter 3), which are possibly selected for by different azoles, which would explain this result.

Considerable knowledge has been gained through recent theoretical modelling of the potential emergence and subsequent selection for resistant or partially resistant strains (Hobbelen *et al*., 2011a, Hobbelen *et al*., 2013, Hobbelen *et al*., 2014, Mikaberidze *et al*., 2014, Van den Bosch *et al*., 2011). Common amongst the predictions of these models has been the usefulness of mixtures of fungicides, whether high-risk:high-risk or high-risk:low-risk combinations, in prolonging the effective life of the most at-risk partner. Unlike these models, our experiments used a mixture of fungicides with medium-resistance-risk and

belonging to the same chemical class. Mixtures are designed to expose the pathogen populations to different modes-of-action, albeit simultaneously rather than sequentially as with alternations. Each component should control a proportion of the strains selected by the other component, thereby reducing the overall selection compared to using a single fungicide. The mixtures provided better disease control but the same yield as the solo products in this experiment. When the effects of treatments on sensitivity were studied for each fungicide separately, the expected positive effect of mixing two components was not seen. Further, when the effects common to both epoxiconazole and metconazole sensitivity were analysed using PCA, the mixtures measured larger shifts in sensitivity than the solo treatments. This increase in selection could simply be due to a further dose effect (Figure 2.6). The pre-formulated mixture used in this experiment contained 90% of the solo epoxiconazole dose and 92% of the solo metconazole dose. If effects are additive this would explain both the improvement in disease control and the absence of a reduction in selection relative to the solo treatments. Interactions between the fungicides in the mixture are likely to have some effect on both disease control and selection, and synergism between the fungicides could explain the improvement in disease control (Kendall & Hollomon, 1994) and the absence of a reduction in selection (Shaw, 1993).

Shaw (1993) suggests that such synergism could be used to reduce selection by using the minimum fungicide dose needed for adequate control. These results contradict this however, as the same size shift was observed after the reduced dose of the mixture as the full dose. From an anti-resistance perspective, this mixture is unusual; it is made up of two azoles which have been shown to select differentially (Fraaije *et al*., 2007), although there is a strong element of cross-resistance shown here by PC1. There is evidence of considerable evolution in the *CYP51* gene (Cools & Fraaije, 2013) and recent work has identified *CYP51* alterations and combinations of alterations which can reduce sensitivity to the majority of azoles, in

particular the S524T mutation (Cools *et al*., 2011) and strains which overexpress the target gene (Cools *et al*., 2012). Additionally, recombination that could bring together alterations conferring reduced sensitivity to each azole (Brunner *et al*., 2008) has been shown in this pathogen. Hence, it is likely that variation exists allowing the azole mixture to select for strains with reduced sensitivity to both fungicides.

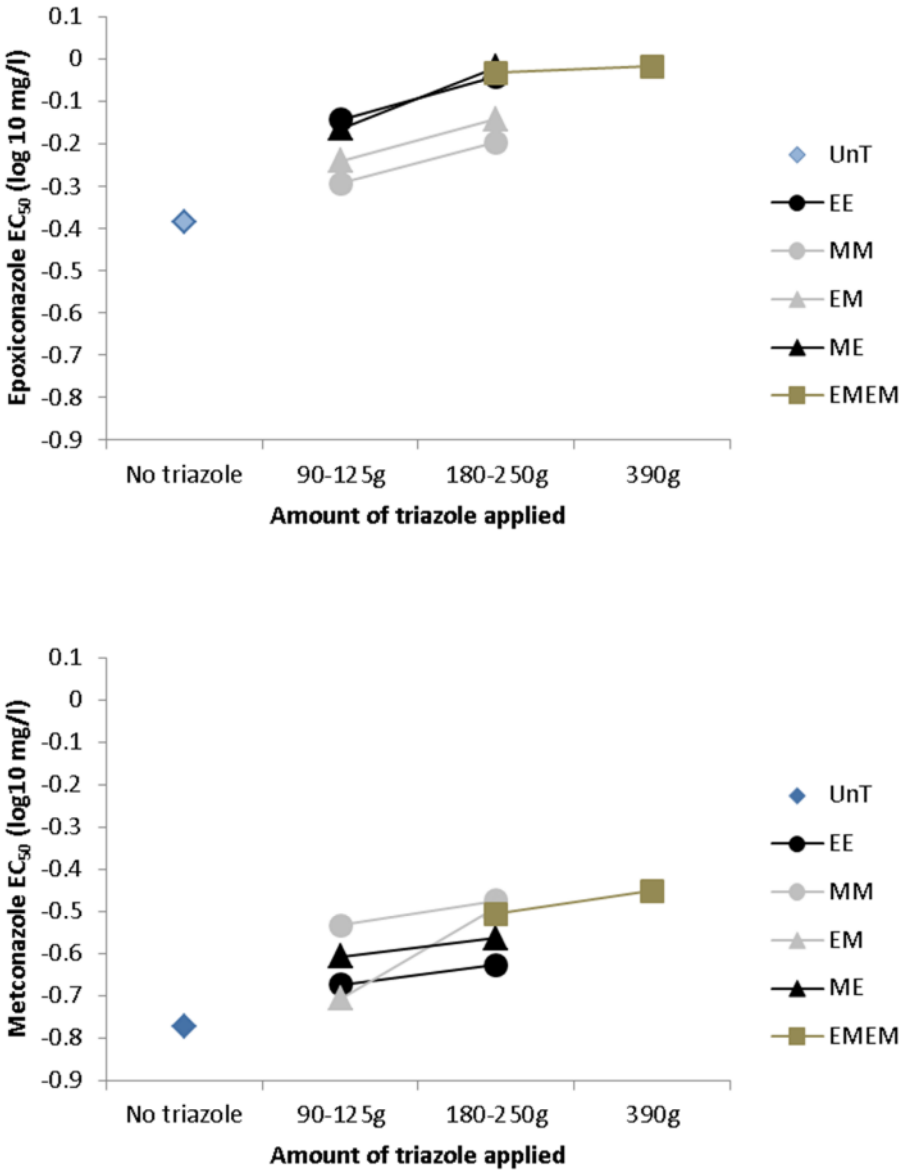


Figure 2.6 Effect of total azole dosage on the sensitivity of epoxiconazole (top) and metconazole (bottom). Treatment information: abbreviations denote the first and second sprays. E: epoxiconazole; M: metconazole; UnT: un-treated control

Limiting the number of applications of an a.i. decreased the selection for strains which are less sensitive to that fungicide. In the treatments where only one application of metconazole was made, i.e. the alternations, the population was significantly more sensitive than the treatments where two applications were made. Even though this was just non-significant at the 5% level for epoxiconazole sensitivity ($P = 0.09$), the same pattern was seen. This supports the prediction in the fungicide resistance model by Hobbelen *et al*. (2011a) in which a significant increase in the selection ratio after an increase in spray numbers was predicted. Applying the same fungicide at each treatment time, i.e. solo treatments and mixtures, keeps the pathogen population under constant selection pressure. Alternations on the other hand allow time between applications of the same fungicide for back-selection of susceptible strains and means the pathogen population is exposed to chemicals exerting different selection pressure at each application time.

The benefit of the two alternations was demonstrated in PC2 of the PCA. Isolates of *Z. tritici* from plots treated with metconazole first and epoxiconazole second were less sensitive to epoxiconazole, and those isolated from plots treated with epoxiconazole first and metconazole second were less sensitive to metconazole, i.e. the most recently applied fungicide had the greatest effect on selection. No comparable findings in an agricultural setting are available. Hobbelen *et al*. (2013) included alternations in their model; however those were fungicides with different modes-of-action. Based on their findings the use of fungicides in alternation is likely to delay the selection for strains with reduced sensitivity, and while different sequences of fungicides were included in the model, this effect of the order of fungicide was not predicted. Results from this experiment are a practical example of what is theoretically expected from combining fungicides for anti-resistance purposes; fungicide A controls the strains sensitive to it while fungicide B controls the strains sensitive to it which happened to be selected by fungicide A and, selects for the strains resistant to it, as

if the population is being pushed one way and then back to keep a balance. However, there is some positive cross-resistance between the two fungicides, so while these push-pull effects should slow the shifts in sensitivity down, it will not completely halt it. There are a number of possible causes of this effect of order of application, which the experimental design does not allow us to distinguish. There could be fitness costs causing back-selection between the first and second applications, so that the second application gave the most response when measured later. There could also be sampling bias, with sampled leaves having been directly treated with the second fungicide but not the first. The effect of dose varied significantly according to the order of application of the two fungicides, but can be explained as an interaction of scale rather than direction. Smaller shifts in sensitivity were observed after both half dose alternations in comparison to their full dose counterparts, but the difference between full and half dose of epoxiconazole followed by metconazole alternation was smaller than the other way round, and probably led to the observed statistical interaction.

Averaged over all application strategies, halving doses did not significantly decrease the shifts in sensitivity. As expected, full doses provided significantly better disease control and, where there was high disease pressure, higher yields. While full recommended doses of fungicide are designed to provide the best possible disease control and are recommended as an anti-resistance strategy by manufacturers, reducing fungicide doses in order to reduce the rate of selection has been argued and tested by numerous researchers; Van den Bosch *et al*. (2011) reviewed the available literature and concluded that all models and most experimental studies show that selection for strains with reduced sensitivity increases with dose. However, the same study also suggested the theory that pathogens which develop a gradual insensitivity, such as *Z. tritici* insensitivity to azoles, may be an exception to the rule, but no evidence was available to add weight to the theory. It is probable that the presence of a large proportion of strains with reduced sensitivity in the population has eroded the efficacy of the fungicides and

that the evolution of azole sensitivity is now in the adjustment phase. So while this study has shown that over all treatments half doses did not increase or decrease sensitivity compared to full doses, and in many individual cases they did increase sensitivity (Table 2.6), the inferior disease control and lower yields after reduced doses makes the strategy used in these experiments impractical. However, in a different situation, such as when fungicides are combined with a different mode-of-action, reduced doses may be more effective .

In conclusion, evidence is presented that limiting the number of applications of an individual a.i. is the most important strategy for managing azole insensitivity; having two azoles which select differentially, and using each sequentially rather than simultaneously, will take advantage of that differential selection to slow down the selection for strains with reduced sensitivity to specific azoles. Disease control achieved by the alternations was the same as that of solo products and control by both was significantly poorer than the mixtures. However, the yields of plots given each application pattern were not significantly different. This strengthens the case for choosing alternations over mixtures or solo products, and emphasises that aiming for perfect disease control may incur costs and increase selection without increasing profit. But how long will that last? The azole mixture here selected for a level of insensitivity to both partners which highlights the need for the inclusion of alternative chemistries in fungicide programs. Reduced doses of azole fungicides were not always effective enough for control of STB. While this conflicts with some other empirical evidence, it shows that the azole insensitive *Z. tritici* population targeted in this experiment is likely to be in the adjustment phase where higher doses are needed for control. Our results demonstrate that anti-resistance recommendations suitable for fungicides with distinct modes-of-action are not always effective when using combinations of azoles. Advice to combine azoles which select for different resistance alleles or loci is vulnerable to continuing genetic change in the pathogen.

Chapter 3: Molecular mechanisms associated with reduced azole sensitivity and the genetic structure of azole treated populations of *Zymoseptoria tritici* in Ireland

3.1 Introduction

Populations of *Zymoseptoria tritici*, the causal agent of Septoria tritici blotch in winter wheat, are characterised by high gene and genotypic diversity (Zhan *et al*., 2006) and combined with its mixed reproductive system and large (effective) population sizes, *Z. tritici* is regarded as having a high evolutionary potential (Zhan & McDonald, 2004). This level of adaptability allows populations of *Z. tritici* to survive and reproduce in new or hostile environments, such as in response to fungicides or resistant hosts. Whilst STB host resistance is available, varieties are generally only moderately resistant (Anon, 2013c). Consequently, multiple well-timed fungicide applications per season are required to control STB. *Zymoseptoria tritici* populations in the major wheat growing regions of the world are seeing shifts in sensitivity to azoles, e.g. in Europe (Leroux *et al*., 2007, Stammler *et al*., 2008, Leroux & Walker, 2011, Stammler & Semar, 2011), Tunisia (Boukef *et al*., 2012) and North America (Estep *et al*., 2014). However, the shift is at different stages in different regions, possibly because of differences in when azoles were introduced and the subsequent level of use, as well as differences in varieties used and agronomic practices.

Previous work has highlighted amino-acid alterations (mutations) in the target protein as the main mechanism which contributes to the reduction in azole sensitivity in *Z. tritici* populations (Cools *et al*., 2005a, Leroux *et al*., 2007, Stammler *et al*., 2008, Cools *et al*., 2010, Cools *et al*., 2011, Cools & Fraaije, 2013). Homology modelling has been used to present a 3D structure of the changes in the target protein that the pathogen goes through during the development of resistance. Resistance to azoles has been studied in this way by Mullins *et al*. (2011) and resistance to SDHIs by Glattli *et al*. (2011), Fraaije *et al*. (2012) and

Scalliet *et al*. (2012). Mullins *et al*. (2011) found that some amino-acid mutations can alter the structure of the binding cavity (region in the protein which is targeted by a specific fungicide molecule (ligand)), but it depends on the location of the altered residue (amino-acid) in relation to the fungicide docking site in the cavity; the closer the mutated amino-acid to the docking site, the more likely it is to interfere with the docking infinity of the fungicide. Figure 3.1 shows where the residues subject to alteration lie in relation to the binding site of the azole, triadimenol. The I381 is very close to the binding cavity and has the effect of reducing the volume of the cavity, possibly bringing remaining residues closer to the binding site. The S188 and N513 residues are just outside of the protein, and far removed from the binding site, meaning that alterations at these residues have less of an impact on the azole binding.

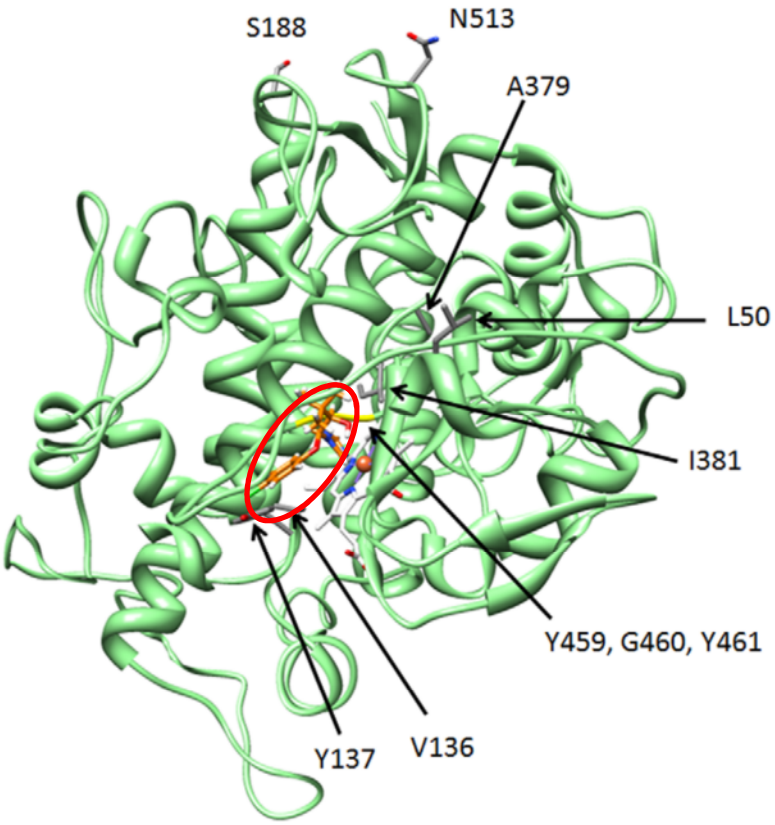


Figure 3.1 Homology model of the wild-type *Z tritici* CYP51, binding triadimenol (circled), courtesy of Mullins *et al*. (2011)

Analysis of *Z. tritici CYP51* target site alterations has confirmed that different azoles select for different alterations (Leroux *et al*., 2007, Fraaije *et al*., 2007), for example, tebuconazole positively selects for the I381V mutation but negatively selects for the V136A mutation. This highlighted the potential of combining differentially selecting azoles as a possible anti-resistance tactic, while maintaining STB control at an acceptable level (Cools & Fraaije, 2008). Recently however, isolates carrying combinations of alterations conferring lab resistance to the most widely used azoles, epoxiconazole and prothioconazole, have emerged (Cools *et al*., 2011), raising concerns that combining azoles might accelerate the development of resistance and loss of azole efficacy in the field. The effect of using combinations of azoles on the selection for insensitive isolates has been covered in more depth in Chapter 2.

Evidence of *Z. tritici CYP51* alterations date from isolates sampled in the early 1990s (Leroux *et al*., 2007, Brunner *et al*., 2008). To date, 34 amino-acid alterations have been reported (Cools & Fraaije, 2013) of which the mutations V136A, A379G, I381V, Y461S, S524T and the double deletion, ΔY459/G460, appear to affect azole sensitivity the most. Leroux *et al*. (2007) studied the genetic characteristics of European *Z. tritici* isolates from between 1988 and 2005. They identified alterations at the 459-461 position in French isolates from as early as 1993. Most of the collections prior to 1997 consisted of wild-type sensitive strains, but from 1997 onwards amino-acid alterations started to accumulate. All combinations of changes in that study showed increasing resistance factors to epoxiconazole and metconazole. In 2007, populations from continental Europe, as well as the UK, were dominated by the I381V mutation and either a mutation or deletion at 459-461 position, and indeed I381V and the double deletion (ΔY459/G460) combined with the A379G mutation was the most common *CYP51* variant in most European countries (Stammler *et al*., 2008). That *CYP51* variant continued to dominate in UK and German populations in 2010, whereas populations in other countries saw a reduction in *CYP51* variants with A379G mutation and

an increase in the frequency of V136C mutation (Stammler & Semar, 2011). Combinations of the V136A and I381V amino-acid mutations were first identified by Stammler *et al*. (2008) in an isolate from 2007, but by 2010 were considerably more frequent in European populations (Stammler & Semar, 2011).

Leroux *et al*. (2007) categorised *Z. tritici* strains according to their sensitivity to specific azoles, and subsequently associated these with changes in their *CYP51*. Earlier *CYP51* variants with few alterations started out as TriS (sensitive) or TriR1-3, which had individual alterations and were slightly less sensitive than TriS. As combinations of alterations which affected sensitivity to specific azoles appeared over time, the number of categories increased and most recently reached TriR12 (V136A, I381V, an alteration at 459-461, and S524T) (Leroux & Walker, 2011). Irish *Z. tritici* strains, compared to strains from other European countries, are relatively advanced along this series; Stammler and Semar (2011) found that two thirds of the Irish population sampled (n = 155) fell into the TriR9 group (V136A, a mutation at 459-461, and S524T) as classified by Leroux & Walker (2011). Since DMI monitoring began around the late 1980s (Leroux *et al*., 2007), Irish *Z. tritici* populations have been somewhat different to the rest of the European populations. Two Irish isolates from 2003 were included in a study by Leroux *et al*. (2007), and they identified the V136A mutation and either a mutation or deletion at 459-461 position in those isolates tested. Research by Kildea (2009) found that the V136A and Y461S mutations were the most common amino-acid alterations, and when combined together was the most frequent *CYP51* variant found in that population. I381V was present but not as common. Leroux and Walker (2011) identified *CYP51* variants in the form of I381V + A379G + ΔY459/G460 and V136A + Y461S + S524T in three Irish isolates from 2009. The latter *CYP51* variant was found to dominate Irish populations in 2010 (Stammler & Semar, 2011), and aside from the UK and Sweden, was not found elsewhere in Europe. Additionally in 2010, isolates with V136A +

Y461S + S524T mutations plus the addition of I381V were identified, albeit in small frequencies. In a more recent but retrospective study, Kildea *et al*. (2014) found the combined V136A + I381V mutations in Irish populations from 2006, and between 2006 and 2011 frequencies of this combination fluctuated.

Recent homology studies (Mullins *et al*., 2011) and heterologous expression of mutated *Z. tritici CYP51* genes (Cools *et al*., 2010) have demonstrated that it is often specific combinations of alterations rather than the individual alterations which have the greatest effect on azole sensitivity. This is also evident in the numerous studies on the development of reduced azole sensitivity in European populations (Cools *et al*., 2005a, Fraaije *et al*., 2007, Leroux *et al*., 2007, Stammler *et al*., 2008, Leroux & Walker, 2011, Stammler & Semar, 2011, Buitrago *et al*., 2014). For a very basic example, Mullins *et al*. (2011) suggest that the early mutation L50S, which has no direct effect on azole sensitivity (Leroux *et al*., 2007), brings the normally deleterious mutations at 136 and 381 into closer proximity with certain azoles, which in turn interferes with the docking ability of the azole, making it less effective. Populations are now significantly more complex, with combinations of many alterations which affect azole sensitivity (Buitrago *et al*., 2014).

In addition to amino-acid alterations in the protein encoded by the *CYP51* gene, other mechanisms are believed to have contributed to the reduction in azole sensitivity in *Z. tritici* (Stergiopoulos *et al*., 2003). Cools *et al*. (2012) have demonstrated that a 120 bp insert in the putative *CYP51* promoter region of field isolates of *Z. tritici* has resulted in the constitutive over-expression of 14α-demethylase, which confers a reduction in sensitivity to all azole fungicides. Chassot *et al*. (2008), Kildea (2009) and Leroux and Walker (2011) have all previously reported a large insert of around 800-1000 bp in the putative *CYP51* promoter region, but whether it was associated with over-expression was unclear (Cools *et al*., 2012).

In comparison to earlier studies of European populations such as Stammler and Semar (2011) and Kildea (2009) who identified eight and nine *CYP51* variants respectively, a recent study by Buitrago *et al*. (2014) identified 38 different genotypes, and included two promoter inserts, 120 bp and 1000 bp, highlighting the extent of increase in *CYP51* diversity in European populations in recent years. To help slow the further development of insensitivity, it may be possible to manipulate selection by making fungicide application decisions based on, amongst other things, knowledge of the current genetic structure of local and regional populations, and of the evolutionary history of these populations. In theory, predictions can be made of how a population will change depending on which active ingredient (a.i.) or combination of a.i.s, are used. In Chapter 2, effects of treatment on azole sensitivity were demonstrated. In the present chapter the hypothesis that treatments have an effect on the variability associated with the *CYP51* gene were tested. The whole *CYP51* gene and the putative *CYP51* promoter region from selected azole treated and un-treated isolates was sequenced and analysed for changes. Further, an evolutionary history of the *CYP51* gene of this *Z. tritici* population is proposed and discussed.

3.2 Materials and methods

3.2.1 Origins of isolates

Zymoseptoria tritici isolates for this study originated from the experiment in Chapter 2, which looked at the effects of combining the azoles epoxiconazole and metconazole on the sensitivity of *Z. tritici* populations. The isolates were selected without knowledge of their genotype, but based on their sensitivity to epoxiconazole and metconazole. Roughly equal numbers of isolates (between 5-7) were chosen from the highest, lowest and intermediate EC_{50} ranges at each location from each of the four treatment groups, pre-treatment, full rate epoxiconazole, full rate metconazole and the full rate mixture of epoxiconazole and

metconazole (see Table 2.1 for location and Table 2.2 for treatment details). EC_{50} boundaries for choosing isolates were not fixed across treatments and locations; they were chosen relative to each treatment at each location. Isolates were retrieved from -80 °C storage and 50 µl of stock spore solutions was spread onto PGA agar to produce spores for DNA extraction.

3.2.2 DNA extraction, amplification and sequencing

Yeasty spores from 5-7 day old cultures of each isolate were freeze dried, homogenised using a bench top mixer mill (Retsch Mixer Mill) and DNA extracted using a GeneElute™ miniprep kit according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA), with the exception of the final elution volume which was adjusted to 150 µl. All DNA was stored at -20 °C. To determine if alterations affecting azole sensitivity were present in the chosen isolates, the entire *CYP51* gene (1907 bp) was amplified by PCR in three overlapping sections (see Table 3.1 for primer details). In some isolates an additional reaction was required to ensure complete sequence coverage of section 1 and 2 (primers F1.5 and R1.5, Table 3.1).

Table 3.1 Polymerase chain reaction (PCR) primers used to amplify the *CYP51* gene in *Z. tritici*

Primers	Sequence	Amplicon size (bp)	*CYP51* region covered
C51-F1 [a]	ACCTGCAGGCAGAACTAAGC	1082	- 160 bp to 922 bp
C51-R1	CCTCCTGTGCCTGACTTCAC		
C51-F1.5 [b]	TGTCCCAATTCGAAGCTCAT	789	415 bp to 1204 bp
C51-R1.5	TGAGCAGCGCAATCATCATA		
C51-F2 [a]	TCGCGGACCTCTACCACTAC	851	741 bp to 1592 bp
C51-R2	GTATTTCTCGGACGGGCTCT		
C51-F3 [a]	GCAAATACAAGGACGGCAAT	944	1154 bp to + 191 bp
C51-R3	GGACAGGATGTGGTCTGGAT		
Mg51-proF [c]	GTGGCGAGGGCTTGACTA	334	
Mg51-seqR	CTGCGCGAGGACTTCCTGGA		

[a] Kildea (Unpublished)
[b] Dooley (Current study)
[c] Cools *et al.*, (2012)

The reaction mixture consisted of 300 nM of both forward and reverse primers, 200 µM dNTPs, 1 X Phusion® buffer (New England BioLabs Inc., Ipswich, MA, USA), 1.5 µl DNA (~ 20 ng), 1 U Phusion® High-Fidelity polymerase (New England BioLabs Inc.), brought to a final reaction volume of 25 µl with molecular grade water. PCR reactions were performed using a Biometra TProfessional Basic Gradient thermal cycler with the following conditions: initial denaturation at 98 °C for 30s, followed by 35 cycles of 98 °C for 10 s, 58 °C for 30 s and 72 °C for 30 s, with a final extension step of 72 °C for 5 min. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and visualised under UV-light (Kodak Image Station). Some isolates did not produce PCR product (see Table 3.2 for details of final isolates). PCR products were sequenced by GATC (Cologne, Germany) and Eurofins MWG Operon (Ebersberg, Germany) using the F1 and R1, F2, F3 and R3 primers for all isolates. The F1.5 primer (Table 3.1) was used on isolates where coverage between sections 1 and 2 was poor.

Table 3.2 Origin of isolates: number of isolates from each treatment; Epox = epoxiconazole; Mixture = epox + metconazole; Met = metconazole; Pre-T = pre-treatment, at each of the six experimental locations in Chapter 2

	Treatment				
Location	Epox	Mixture	Met	Pre-T	Total per location
Duleek	18	14	16	18	66
Julienstown	14	18	15	14	61
Killeagh	17	18	18	18	71
Knockbeg	19	20	16	16	71
Oak Park	13	18	15	13	59
Stamullen	14	18	16	17	65
Total per treatment	95	106	96	96	Grand total (393)

3.2.3 Analysis of the putative *CYP51* promoter region

The primers Mg51-proF and Mg51-seqR (Table 3.1) from Cools *et al*. (2012) were used to amplify the sections of DNA upstream from the start site of the *CYP51* gene. The reaction

mixture consisted of 300 nM of both forward and reverse primers, 200 µM dNTPs, 1 X ThermoPol™ Buffer (New England BioLabs Inc.), 1.5 µl DNA (~ 20 ng), 1 U *Taq* polymerase (New England BioLabs Inc.), brought to a final reaction volume of 25 µl with molecular grade water. PCR reactions were performed using the following conditions: initial denaturation at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final incubation at 72 °C for 5 min. Deviations from the expected 334 bp amplicons indicated an indel event.

3.2.4 Data analysis

Sequence data for each isolate were assembled using CLC genomics workbench v5.1 (www.clcbio.com [Accessed 28-12-14]), edited and aligned with sequence data from the wild-type *CYP51* gene ST1 (Accession no. AY730587 http://www.ncbi.nlm.nih.gov/nuccore/AY730587 [Accessed 28-12-14]) using the Clustal W function in BioEdit v7.0.9.0 (Hall, 1999) (http://www.mbio.ncsu.edu/bioedit/bioedit.html [Accessed 28-12-14]). Introns were removed from the sequence. Nucleotide sequences (all synonymous and non-synonymous substitutions) were used for the study of the overall genetic diversity of the population and evolutionary history of the isolates, but translated to amino-acids (non-synonymous substitutions only) for the study of the *CYP51* variants. Synonymous substitutions are changes to the nucleotide sequence which have no effect on the protein, and non-synonymous substitutions change the protein.

A chi-square test was used to associate frequencies of individual amino-acid alterations with treatments and with geographic locations, and to identify possible interactions between treatment and location. Amino-acid alterations which were found at least once at every location and in every treatment were included in this analysis which was performed using a generalised linear model with a Poisson distribution and log link function in GenStat v14.1.0. Rarefaction analysis was used to correct for uneven sample sizes when analysing

CYP51 variant diversity between locations, and between treatments. Population differentiation (G_{st}, (Nei, 1973)) was measured between locations and between treatment groups, and notional amount of gene flow (N_m, (Boeger *et al*., 1993)) was measured between geographic locations using the gene flow and genetic differentiation function in DnaSP v5.10.01 (http://www.ub.edu/dnasp/ [Accessed 28-12-14]) (Librado & Rozas, 2009). Effects of all amino-acid alterations (including the presence of an insert in the promoter; a combined factor for rare alterations seen less than 5 times; and a combined factor for all alterations at 136 position) and all combinations of alterations, on epoxiconazole and metconazole sensitivity measured on a log concentration scale, were tested using a generalised linear model in GenStat, with normal error distribution. The models were simplified by dropping all non-significant interactions, after which all non-significant main effects were also removed.

Logistic regression, carried out in GenStat, was used to analyse the effect of treatment on frequencies of *CYP51* variants; only variants which were seen at least once in each treatment group were analysed. The effects of *CYP51* variants on fungicide sensitivity were measured using the linear mixed model (REML) function in GenStat: in the model, *CYP51* variant (49 levels) was considered a fixed effect, whilst isolate was considered random effect. The REML function in GenStat was also used to test the association between fungicide sensitivity and *CYP51* variants split with/without the ~800 bp insert: in the model, the split variants were considered a fixed effect (12 levels) whilst isolate was considered random effect.

Isolates and *CYP51* variants were grouped according to sensitivity of the whole collection where $\log_{10}EC_{50}$ values of below -0.3 mg L^{-1} (0.5 mg L^{-1}) for epoxiconazole and $\log_{10}$ -0.523 mg L^{-1} (0.3 mg L^{-1}) for metconazole were considered to be sensitive, $\log_{10}EC_{50}$ values of above 0.176 mg L^{-1} (1.5 mg L^{-1}) for epoxiconazole and 0.0 mg L^{-1} (1.0 mg L^{-1}) for metconazole were considered to be insensitive, and the remainder were considered to be

moderately sensitive. Phenotypes were visually analysed, using boxplots and variant means, and interesting features discussed.

Evolutionary analysis of the data set began with the construction of a maximum likelihood (ML) tree using MEGA v6.0 (http://www.megasoftware.net/) (Tamura *et al.*, 2013). All analyses were performed using the Kimura 2-parameter model. The tree with the highest log likelihood was shown. A bootstrap analysis was performed to test for statistical significance of the trees generated with 200 pseudoreplications. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.1000)). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Because recombination cannot be identified in the ML tree, a median-joining network was constructed using NETWORK v4.6.1.2 (http://www.fluxus-engineering.com/sharenet.htm [Accessed 28-12-14]) to identify whether recombination was likely to be present in the collection. Specific recombination break points (RBP) were highlighted using the GARD (a genetic algorithm for recombination detection) function in the open source HyPhy software found at DataMonkey.org (http://www.datamonkey.org/ [Accessed 28-12-14]). New phylogenetic trees were created using MEGA v6.0 with sequence data from the left and from the right of the potential RBP; tree topologies were compared, and recombination was inferred when tree topologies were significantly different. Visual analysis of the trees highlighted which haplotypes are recombinants; haplotypes which have switched clades in the tree to the right of the RBP are identified as potential recombinants. Potential recombinants were removed from the data set and a new ML tree created in MEGA v6.0.

3.3 Results

3.3.1 Brief description of the collection

The 1907 bp *CYP51* gene of each isolate was sequenced, of which a 1635 bp sequence (introns removed) was screened for changes compared to the wild-type strain ST1. A total of 50 polymorphic sites were identified, where non-synonymous substitutions leading to 21 amino-acid alterations at 17 positions throughout the protein were found. There were 49 different combinations of mutations (*CYP51* variants, Table 3.3). In addition, a 6 bp-deletion leading to the deletion of two amino-acids (ΔY459/G460) was found. No wild-type sequences were found, and all isolates had at least one amino-acid alteration previously associated with a decrease in azole sensitivity (Cools & Fraaije, 2013). The most commonly observed alterations were L50S, I381V, V136A and S524T, found in 99%, 66%, 55% and 54% of the isolates respectively (Table 3.4). Two amino-acid mutations were novel; I377V and I384M. In addition, three different size inserts were found in the collection following PCR amplification of the putative promoter region (Figure 3.2). A single isolate had no PCR product. A total of 195 isolates had the expected product size of 334 bp, i.e. no insert present;

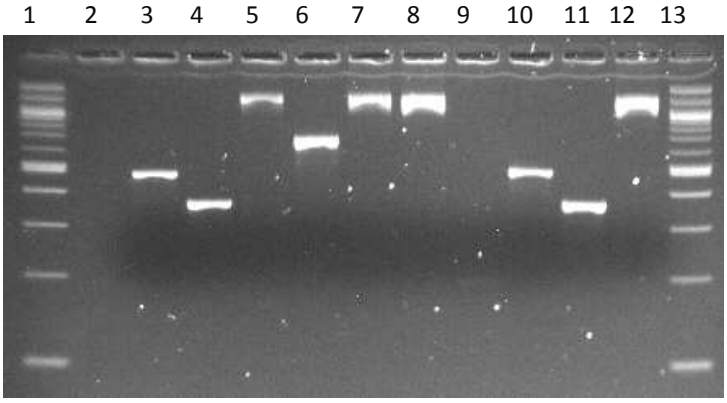


Figure 3.2 Gel electrophoresis showing the four PCR products found in the collection from amplifying the regulatory sequences upstream of *CYP51*. 100 bp ladder in lane one and lane 13. Lanes 4 and 11 have the expected product size of 334 bp; lanes 3 and 10 have the 120 bp insert; lanes 5, 7, 8 and 12 have the ~800 bp insert and lane 6 has the ~300 bp insert. (Image courtesy of J M-C, Teagasc)

43 isolates had the 120 bp insertion as identified by Cools *et al*. (2012); a single isolate had an insert with an estimated size of ~300 bp; and the remaining 153 isolates had an insert of approximately ~800 bp (see Table 3.4 and Table 3.8 for frequencies of each amino-acid alteration and inserts at each location and treatment).

3.3.2 Effect of *CYP51* variants on sensitivity

The collection of isolates consisted of 49 unique combinations of amino-acid alterations (six of which split into two groups, a and b, to represent the presence or absence of the ~800 bp insert), ranging in frequency from 1 to 97 (n = 393, Table 3.3). The nine most frequent *CYP51* variants represent 80% of all isolates, and approximately half of all *CYP51* variants were only seen in single isolates. *CYP51* variants had between three and eight alterations. Sensitivity tended to decrease as the number of alterations per *CYP51* variant increased (Table 3.3). Even though the range of EC_{50} values for many *CYP51* variants overlapped (Figure 3.3), there was a significant effect of *CYP51* variant on the sensitivity to both epoxiconazole and metconazole (REML, $P < 0.001$ for both fungicides).

Isolates with the 120 bp insert were found in five different *CYP51* variants and whilst there were differences in the mean sensitivity between these variants (Table 3.3), 65% of the individual isolates from those *CYP51* variants were classed in the insensitive categories of both epoxiconazole and metconazole. Within the isolate collection, the 120 bp insert did not appear in a common background to any *CYP51* variants without the insert, so it was not possible to compare the effects of that insert on sensitivity. The larger insert of ~800 bp was found in 24 *CYP51* variants, including variants 2, 4, 31, 35, 37 and 38, where there was a mix of isolates with and without the insert. In the two *CYP51* variants 31 and 35 (where there were a sufficient number of isolates to compare the effects on sensitivity between those with and without the insert.) the ~800 bp insert significantly reduced sensitivity to both fungicides (REML, $P < 0.001$, Figure 3.4).

The most common *CYP51* variant, variant 11 (alterations L50S, V136A, Y461S and S524T, Table 3.3) was found in 25% of the whole collection and in each treatment and location. Isolates with that *CYP51* variant were on average categorised as sensitive, but some individual isolates were moderately sensitive and a few were insensitive (Figure 3.3). A total of seventeen *CYP51* variants were categorised as sensitive to both epoxiconazole and metconazole (Table 3.3). Most of these *CYP51* variants had no more than four amino-acid alterations and no isolates with the V136A/I381V combination were present in this category. Five *CYP51* variants (variants 25, 26, 38, 44, 48) had isolates which were mostly insensitive to both fungicides (Table 3.3), even though some isolates with these *CYP51* variants were sensitive (Figure 3.3). All *CYP51* variants with this insensitive phenotype had accumulated five or more alterations, and the V136A/I381V combination was seen in one of these (variant 38). Two *CYP51* variants were, on average, moderately sensitive to both fungicides (variants 29 and 34). The remaining *CYP51* variants had different phenotypes for each fungicide (Table 3.3). *CYP51* variant 9 was very insensitive to epoxiconazole but very sensitive to metconazole; it carried L50S, D134G, V136G and Y461S alterations (Table 3.3).

Table 3.3 *CYP51* variants in the current collection. Each *CYP51* variant represents a different combination of amino-acid alterations, and *N* the number of times that *CYP51* variant was found. Treatment illustrates the treatments from which *CYP51* variants were found; E = epoxiconazole; M = metconazole; G = the mixture; P = pre-treatment. Location illustrates the location in which *CYP51* variants were found; D = Duleek; K = Knockbeg; S = Stamullen; J = Julienstown; C = Killeagh; and O = Oak Park

Variant name	*N*	Amino-acid changes in each *CYP51* variant	Insert	Mean $\log_{10}EC_{50}$ (mg L^{-1})				Treatment	Location
				Epoxiconazole		Metconazole			
1	1	L50S, S188N, I381V	120	-0.648		-1.223		E	S
2	6	L50S, V136A, Y461S	800*	-0.982	± 0.102	-1.443	± 0.15	P, E	D, J, K, S
2a	*5*	*L50S, V136A, Y461S*	*0*	*-1.000*	*± 0.123*	*-1.500*	*± 0.169*	*P, E*	*D, J, K, S*
2b	*1*	*L50S, V136A, Y461S*	*800*	*-0.889*		*-1.157*		*P*	*K*
3	16	L50S, V136C, Y461S	0	-0.729	± 0.069	-0.855	± 0.083	P, E, G, M	D, J, K, O, S
4	13	L50S, I381V, Y461H	800*	-0.896	± 0.114	-1.015	± 0.096	P, E, G, M	D, J, K, O, S
4a	*2*	*L50S, I381V, Y461H*	*0*	*-0.896*	*± 0.114*	*-1.036*	*± 0.283*	*E, G,*	*O, S*
4b	*11*	*L50S, I381V, Y461H*	*800*	*-0.891*	*± 0.136*	*-1.012*	*± 0.108*	*P, E, G, M*	*D, J, C, K, O, S*
5	3	L50S, I381V, Y459S	0	-0.2	± 0.585	-0.801	± 0.042	P, E, G	D, O
6	2	L50S, I381V, Y459D	0	-0.753	± 0.153	-0.868	± 0.059	P, G	C, K
7	1	L50S, Y461S, S524T	0	-0.741		-1.439		E	J
8	1	L50S, Y461S, V490L	0	-1.096		-0.682		P	K
9	1	L50S, D134G, V136G, Y461S	0	0.610		-0.719		E	D
10	5	L50S, V136A, Y461H, S524T	0	-0.621	± 0.152	-1.233	± 0.182	E, G, M	D, K, S
11	97	L50S, V136A, Y461S, S524T	0	-0.440	± 0.032	-0.946	± 0.043	P, E, G, M	D, J, C, K, O, S
12	1	L50S, V136C, Y461S, S524T	0	-0.308		-0.353		G	K
13	3	L50S, S188N, I381V, Y461S	120	-0.397	± 0.315	-0.670	± 0.467	M	D
14	2	L50S, S188N, I381V, Y461H	120	0.027	± 0.648	0.236	± 0.414	G	S
15	1	L50S, A311G, Y461S, V490L	0	-0.918		-0.655		M	O
16	3	L50S, I377V, I381V, Y461H	800	-0.543	± 0.049	-0.732	± 0.032	P, E, G	C, O
17	1	L50S, I381V, Y461S, S524T	0	-0.757		-1.264		E	J
18	1	L50S, A379G, I381V, Del, N513K	0	-0.759		-0.106		P	K

Table 3.3 cont.

Variant name	*N*	Amino-acid changes in each *CYP51* variant	Insert	Mean $log_{10}EC_{50}$ (mg L^{-1})				Treatment	Site
				Epoxiconazole		Metconazole			
19	1	L50S, Y461S, V490L, S524T	0	-1.088		-0.571		M	K
20	4	D107V, I381V, N513K, S524T	0	-1.045	± 0.058	-1.127	± 0.074	P, E, G	D, O, S
21	1	L50S, D134G, V136A, I381V, Y461S	800	-0.160		-0.916		E	D
22	33	L50S, D134G, V136A, I381V, Y461H	800	-0.161	± 0.497	-0.811	± 0.051	P, E, G, M	D, J, C, K, O, S
23	2	L50S, D134G, V136G, Y461S, S524T	0	0.670	± 0.244	-0.134	± 0.135	E	D, S
24	1	L50S, S188N, A379G, I381V, Y461S	800	0.054		0.097		G	K
25	34	L50S, S188N, I381V, Del, N513K	120	0.317	± 0.067	0.037	± 0.063	P, E, G, M	D, J, O, S
26	3	L50S, S188N, I381V, Y461S, S524T	120	0.251	± 0.227	0.306	± 0.055	E	S
27	1	L50S, S188N, I377V, I381V, Y461H	0	-0.257		-0.639		M	J
28	1	L50S, V136A, S188N , I381V, Y461H	0	0.126		-0.657		M	J
29	1	L50S, V136A, S188N, Del, S524T	0	-0.208		-0.520		P	O
30	1	L50S, V136A, S188N, Del, N513K	0	-0.558		-1.035		P	O
31	41	L50S, V136A, I381V, Y461S, S524T	800*	0.413	± 0.066	-0.498	± 0.052	P, E, G, M	D, J, C, K, O, S
31a	*23*	*L50S, V136A, I381V, Y461S, S524T*	*0*	*0.160*	*± 0.064*	*-0.680*	*± 0.056*	*P, E, G, M*	*D, J, C, O, S*
31b	*18*	*L50S, V136A, I381V, Y461S, S524T*	*800*	*0.737*	*± 0.075*	*-0.266*	*± 0.059*	*E, G, M*	*D, J, C, K, O, S*
32	16	L50S, V136A, I381V, Y461H, S524T	800	0.681	± 0.107	-0.364	± 0.072	P, E, G, M	D, J, C, K, O
33	1	L50S, V136C, I381V, Y461S, S524T	0	0.967		-0.071		E	J
34	2	L50S, D134G, V136A, I381V, Y461H, S524T	800	0.086	± 0.034	-0.508	0.136	G	D, K
35	52	L50S, S188N, A379G, I381V, Del, N513K	800**	-0.604	± 0.053	-0.49	± 0.058	P, E, G, M	J, C, K, O, S
35a	*19*	*L50S, S188N, A379G, I381V, Del, N513K*	*0**	*-0.767*	*± 0.085*	*-0.701*	*± 0.064*	*P, E, G, M*	*C, K, O, S*
35b	*33*	*L50S, S188N, A379G, I381V, Del, N513K*	*800**	*-0.51*	*± 0.064*	*-0.369*	*± 0.077*	*P, E, G, M*	*J, C, K, O, S*
36	10	L50S, S188N, A379G, I381V, Del, S524T	800	0.082	± 0.159	0.180	± 0.134	E, G, M	D, J, C, K, O, S

Table 3.3 cont.

Variant name	*N*	Amino-acid changes in each *CYP51* variant	Insert	Mean $log_{10}EC_{50}$ (mg L^{-1})				Treatment	Site
				Epoxiconazole		Metconazole			
37	6	L50S, S188N, A379G, I381V, Y461S, S524T	800*	-0.387	± 0.206	-0.350	± 0.24	G, M	J, K
37a	*2*	*L50S, S188N, A379G, I381V, Y461S, S524T*	*0*	*-0.783*	*± 0.22*	*-0.961*	*± 0.153*	*G, M*	*J, K*
37b	*4*	*L50S, S188N, A379G, I381V, Y461S, S524T*	*800*	*-0.189*	*± 0.243*	*-0.045*	*± 0.217*	*G, M*	*J, K*
38	3	L50S, V136A, A379G, I381V, Y461S, S524T	800*	0.629	± 0.324	0.042	± 0.147	E, G	D
38a	*2*	*L50S, V136A, A379G, I381V, Y461S, S524T*	*0*	*0.328*	*± 0.207*	*0.036*	*± 0.255*	*E, G*	*D*
38b	*1*	*L50S, V136A, A379G, I381V, Y461S, S524T*	*800*	*1.231*		*0.053*		*G*	*D*
39	1	L50S, A379G, I381V, Del, N513K, S524T	800	-0.076		0.107		M	J
40	1	L50S, D134G, V136A, A379G, I381V, Y461S, S524T	800	1.383		-0.285		E	O
41	1	L50S, S188N, N284H, A379G, I381V, Del, N513K	0	0.323		-0.115		M	C
42	1	L50S, S188N, A379G, I381V, I384M, Del, N513K	800	-0.703		-0.125		P	S
43	1	L50S, S188N, A379G, I381V, A410S, Del, N513K	0	-0.201		0.196		E	C
44	4	L50S, S188N, A379G, I381V, Del, N513K, S524T	800	0.245	± 0.264	0.364	± 0.168	G, M	D, C, K, O
45	1	L50S, S188N, A379G, I381V, Y461S, N513K, S524T	800	-0.336		-1.067		M	K
46	3	L50S, V136A, S188N, A379G, I381V, Y461S, S524T	0	0.636	± 0.163	-0.049	± 0.134	P, E, G	D, O
47	6	L50S, V136A, S188N, A379G, I381V, Del, S524T	800	0.715	± 0.137	-0.127	± 0.012	E, G, M	J, C, O
48	1	L50S, V136C, S188N, A379G, I381V, Del, S524T	800	1.176		0.775		E	C
49	1	L50S, V136A, S188N, A379G , I381V, Del, N513K, S524T	800	0.986		-0.012		G	K

* *CYP51* variants which are split by the presence or absence of the ~800 bp insert, underneath each of these variants and in italics is the breakdown of information of the split variants, with and without the ~800 bp insert.

The colours highlighting the mean EC_{50} values represent the phenotypic classification each *CYP51* variant falls into. Green = sensitive: $log_{10}EC_{50}$ values of below -0.3 mg L^{-1} for epoxiconazole and log_{10} -0.523 mg L^{-1} for metconazole, Red = insensitive: $log_{10}EC_{50}$ values of above 0.176 mg L^{-1} for epoxiconazole and 0.0 mg L^{-1} for metconazole, Orange = moderately sensitive: $log_{10}EC_{50}$ values between sensitive and insensitive.

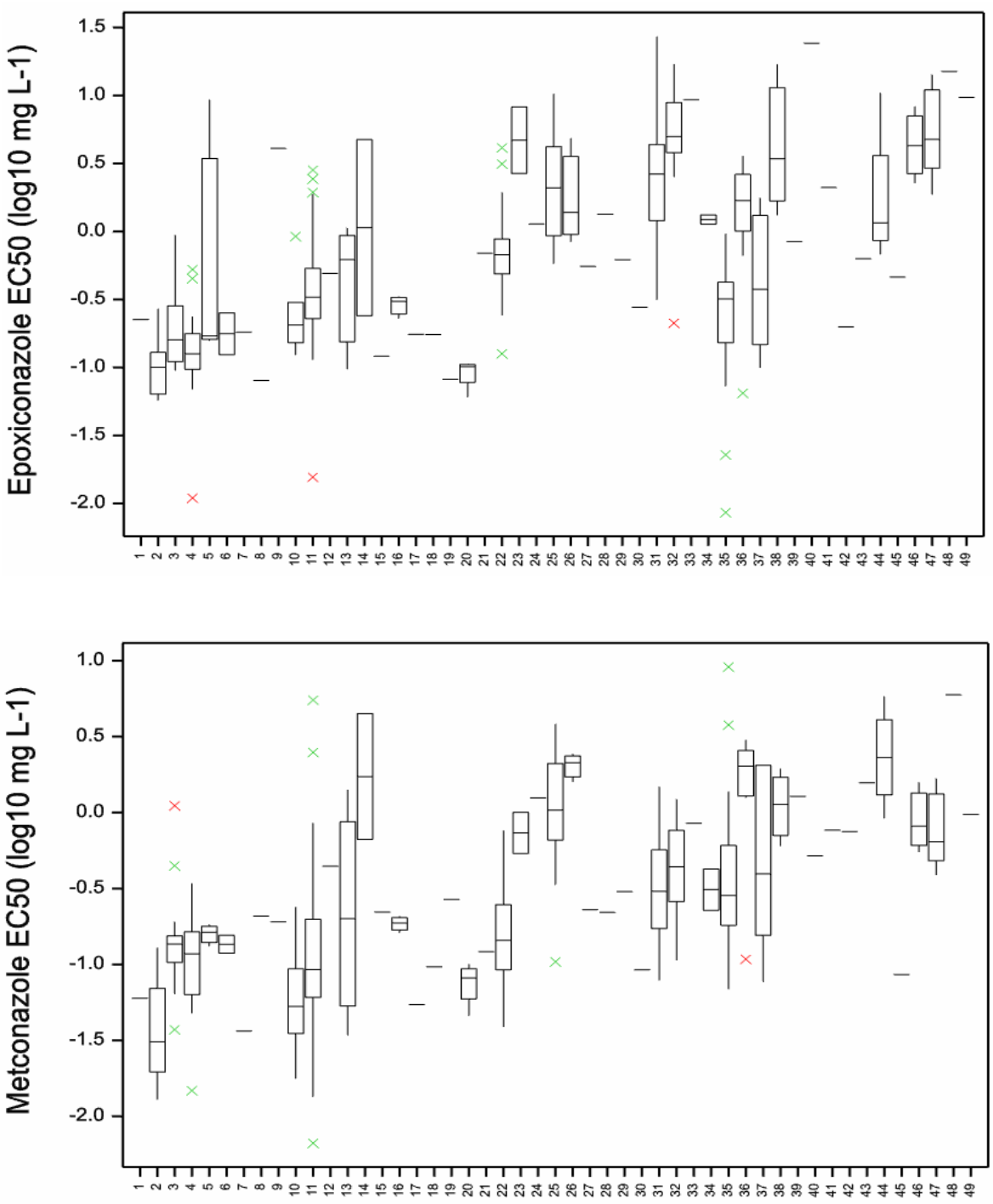


Figure 3.3 Sensitivity, measured as $log_{10}EC_{50}$ mg L^{-1}, to epoxiconazole (top) and metconazole (bottom) of isolates in all *CYP51* variants. *CYP51* variants with one isolate only have a single dash with no box & whisker. For *CYP51* variants with more than one isolate, the line through the box represents the median, and x represents an outlier. See Table 3.3 for *CYP51* variant details. Sensitivity categories: sensitive; as $log_{10}EC_{50}$ values of below -0.3 mg L^{-1} for epoxiconazole and -0.523 mg L^{-1} for metconazole, insensitive; as $log_{10}EC_{50}$ values of above 0.176 mg L^{-1} for epoxiconazole and 0.0 mg L^{-1} for metconazole, and moderately sensitive: $log_{10}EC_{50}$ values between sensitive and insensitive

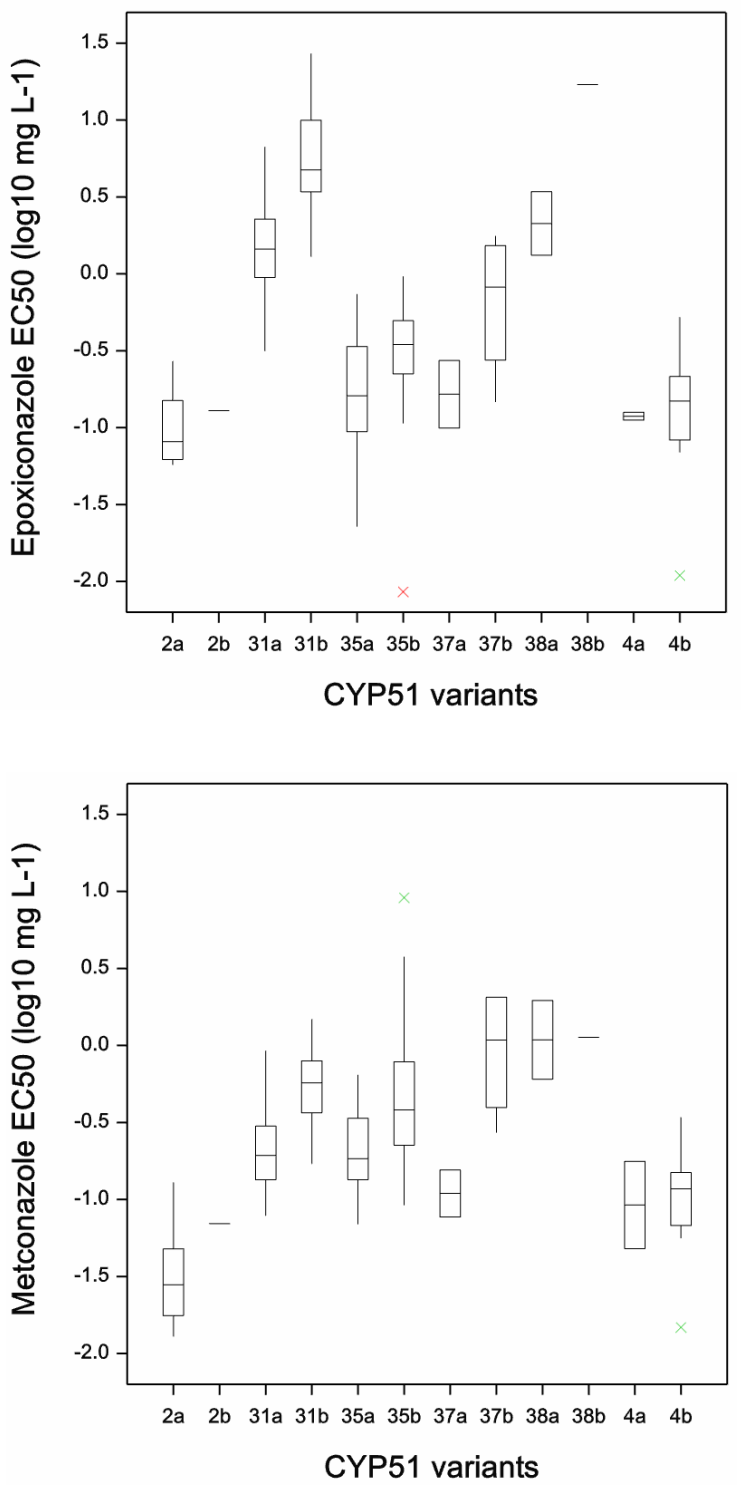


Figure 3.4 Sensitivity to epoxiconazole (top) and metconazole (bottom) of isolates in *CYP51* variants, split into groups with (a) and without (b) the ~800 bp insertion. *CYP51* variants with only one isolate have no box & whisker. For *CYP51* variants with more than one isolate, the line through the box represents the median, and x represents an outlier. See Table 3.3 for *CYP51* variant details

3.3.3 Effect of location on amino-acid and insert frequencies, and genetic diversity

There were significant associations between the frequency of some amino-acid alterations/inserts and geographic location (Table 3.4). The S188N, N513K and ΔY459/G460 alterations varied in frequency patterns between sites in a similar way, all three were seen most frequently at Killeagh and Stamullen, and least frequently at Duleek. Patterns of frequency of Y461S and S524T were also similar; they were seen most frequently at Duleek and least frequently at Killeagh. I381V was mostly found at Killeagh and was found least often at Duleek. V136A was found most frequently at Duleek and least frequently at Stamullen. The 120 bp insert was found at four sites with the highest frequency at Stamullen (n = 25) and the lowest at Oak Park (n = 1). The ~800 bp insert however was found least frequently at Stamullen and most frequently at Killeagh and Knockbeg.

High levels of genotypic diversity were seen at each geographic location (Table 3.5). Between 15 and 20 *CYP51* variants were found at each geographic location (Table 3.5), many of which were common between locations (Table 3.6). Killeagh had the least *CYP51* variant diversity, followed by Stamullen, with the remaining four locations having higher and similar levels of *CYP51* variant diversity. Rarefaction analysis demonstrated that the pattern of *CYP51* variant diversity between locations would have been similar if sample sizes were equal (Table 3.5). Genetic differentiation between locations was low, with G_{st} values of between 0.001 and 0.07 (Table 3.7), and equivalent gene flow between locations was therefore high, N_m of between 3 and 127 (Table 3.7). Gene flow was highest between Oak Park and Knockbeg (N_m = 127) and lowest between Killeagh and Duleek/Julienstown (N_m = 3).

Table 3.4 Number of times amino-acid alterations and promoter insert were found in the whole collection, and at individual geographical locations, represented as percentage of location sample size

Amino-acid alteration	Overall frequency (n = 393)	Location						Chi-square[a]	
		Duleek (n = 66)	Julienstown (n = 61)	Killeagh (n = 71)	Knockbeg (n = 71)	Oak Park (n = 59)	Stamullen (n = 65)	X^2_5	*P*
L50S	99%	98%	100%	100%	100%	98%	97%		
D107V	1%	2%	0%	0%	0%	2%	3%		
D134G	10%	8%	3%	18%	14%	14%	3%	15.5	0.008
V136A	55%	77%	57%	46%	49%	64%	40%	25.3	< 0.001
V136C	5%	3%	8%	1%	10%	2%	5%	8.9	0.11
V136G	1%	3%	0%	0%	0%	0%	2%		
S188N	34%	12%	30%	55%	30%	31%	48%	36.4	< 0.001
N284H	0%	0%	0%	1%	0%	0%	0%		
A311G	0%	0%	0%	0%	0%	2%	0%		
I377V	1%	0%	2%	3%	0%	2%	0%		
A379G	24%	9%	8%	55%	31%	27%	9%	Site.Treatment interaction[b]	
I381V	66%	44%	72%	87%	63%	61%	66%	32.3	< 0.001
I384M	0%	0%	0%	0%	0%	0%	2%		
A410S	0%	0%	0%	1%	0%	0%	0%		
Y459D	1%	0%	0%	1%	1%	0%	0%		
Y459S	1%	3%	0%	0%	0%	2%	0%		
Y461H	19%	12%	13%	24%	31%	25%	9%	Site.Treatment interaction	
Y461S	49%	77%	61%	20%	46%	44%	48%	52.6	< 0.001

Table 3.4 cont.

Amino-acid alteration	Overall frequency	Location						Chi-square[a]	
		Duleek (n = 66)	Julienstown (n = 61)	Killeagh (n = 71)	Knockbeg (n = 71)	Oak Park (n = 59)	Stamullen (n = 65)	X^2_5	*P*
Del[c]	29%	6%	26%	55%	21%	27%	38%	48.2	< 0.001
V490L	1%	0%	0%	0%	3%	2%	0%		
N513K	26%	6%	23%	44%	21%	24%	37%	33.4	< 0.001
S524T	54%	79%	61%	35%	46%	56%	51%	30.8	< 0.001
120 bp	11%	8%	20%	0%	0%	2%	38%	78.3	< 0.001
~300 bp	0%	0%	0%	1%	0%	0%	0%		
~800 bp	39%	17%	34%	66%	55%	44%	14%	68	< 0.001

[a]The Chi square statistic shows the P-value for the association between the locations sampled and the occurrence of the more common amino-acid alterations and promoter inserts in *Zymospetoria tritici* isolates

[b]The site.treatment interaction highlights the amino-acid alterations/promoter inserts where the chi square results for the associations with treatments (Table 3.8) differed significantly between locations

[c]Del = ΔY459/G460

Table 3.5 Summary statistics of molecular diversity found at the *CYP51* gene in *Zymoseptoria tritici* isolates collected from six different geographical locations

Location	N	S	h	Hd	*SS*	*NSS**	*Var*	*RareFn*	*NSS/SS*
Duleek	66	42	54	0.803	25	14 Del	20	19	0.56
Knockbeg	71	35	24	0.915	19	13 Del	20	17	0.68
Stamullen	65	39	17	0.851	22	14 Del	16	15	0.63
Julienstown	61	34	18	0.855	19	12 Del	18	17	0.63
Killeagh	71	36	17	0.807	18	15 Del	15	13	0.83
Oak Park	59	41	19	0.855	21	16 Del	19	19	0.76

N = sample size
S = number of segregating (polymorphic) sites (incl 6 bp deletion)
h = number of nucleotide haplotypes
Hd = haplotype diversity
SS = number of synonymous substitutions
NSS * = number of non-synonymous substitutions and deletion
Var = actual number of *CYP51* variants
RareFn: Potential numbers of *CYP51* variants if all sample sizes were equal to the smallest current sample size

Table 3.6 Proportion of common haplotypes between geographic location, and unique haplotypes within geographic location (in bold numbers)

Location	Duleek	Knockbeg	Stamullen	Julienstown	Killeagh	Oak Park
Duleek	**0.24**					
Knockbeg	0.65	**0.3**				
Stamullen	0.65	0.45	**0.25**			
Julienstown	0.53	0.56	0.5	**0.33**		
Killeagh	0.41	0.6	0.47	0.44	**0.27**	
Oak Park	0.71	0.45	0.47	0.56	0.67	**0.21**

Table 3.7 Pairwise comparisons of gene flow (N_m) (above the diagonal) and population differentiation (G_{st}) (below the diagonal) in the *Zymoseptoria tritici* populations from the six geographic locations

Location	Duleek	Knockbeg	Stamullen	Killeagh	Julienstown	Oak Park
Duleek	*	8	8	3	8	17
Knockbeg	0.03	*	7	15	7	127
Stamullen	0.03	0.03	*	3	35	8
Killeagh	0.07	0.02	0.07	*	4	11
Julienstown	0.03	0.03	0.007	0.06	*	7
Oak Park	0.01	0.001	0.03	0.02	0.03	*

3.3.4 Effect of treatment on amino-acid and insert frequencies and genetic diversity

Although the isolates studied were selected based on sensitivity within site-treatment collections, and so it is a non-random sample, it is worth noting the following observations. In comparison to the pre-treatment collection, the S188N, N513K and ΔY459/G460 alterations were found more commonly after the metconazole and mixture treatments, but less often after epoxiconazole. The S524T and D134G alterations increased in frequency after the epoxiconazole and mixture treatments but decreased after metconazole. Y461S decreased after the metconazole and mixture treatments but stayed the same after epoxiconazole. V136A increased after epoxiconazole and the mixture but decreased after metconazole (Table 3.8). I381V increased after all treatments, but more so after the mixture. The 120 bp insert increased after the mixture and metconazole treatments and the ~800 bp increased after all treatments, but mostly after the mixture. The frequency of A379G and Y461H alterations were different after each treatment depending on the location. In Killeagh and Knockbeg, where the A379G and Y461H alterations were more common than at other locations, A379G was more frequent after the metconazole and mixture treatments and Y461H was most frequently found after the epoxiconazole treatment.

Table 3.8 Number of times amino-acid alterations, and promoter insert were found in the whole collection, classified and split by treatment

Amino-acid alteration	Overall frequency (n = 393)	Treatment				Chi-Square[a]	
		Pre-T (n = 96)	Epox (n = 95)	Mixture (n = 106)	Met (n = 96)	X^2_3	*P*
L50S	99%	99%	99%	98%	100%		
D107V	1%	1%	1%	2%	0%		
D134G	10%	8%	17%	12%	3%	11.8	0.008
V136A	55%	60%	73%	52%	38%	25.9	< 0.001
V136C	5%	8%	3%	2%	6%	5.7	0.125
V136G	1%	0%	3%	0%	0%		
S188N	34%	20%	17%	45%	54%	45.6	< 0.001
N284H	0%	0%	0%	0%	1%		
A311G	0%	0%	0%	0%	1%		
I377V	1%	1%	1%	1%	1%		
A379G	24%	17%	14%	29%	35%	Site.Treatment interaction[b]	
I381V	66%	45%	68%	79%	70%	27.9	< 0.001
I384M	0%	1%	0%	0%	0%		
A410S	0%	0%	1%	0%	0%		
Y459D	1%	1%	0%	1%	0%		
Y459S	1%	1%	1%	1%	0%		
Y461H	19%	19%	26%	22%	10%	Site.Treatment interaction	
Y461S	49%	58%	58%	36%	45%	14.5	0.002
Del[c]	29%	20%	13%	39%	45%	34	< 0.001
V490L	1%	1%	0%	0%	2%		
N513K	26%	21%	11%	31%	41%	26.7	< 0.001
S524T	54%	48%	68%	54%	47%	11.6	0.009
120 bp	11%	3%	5%	17%	18%	19.3	< 0.001
~300 bp	0%	1%	0%	0%	0%		
~800 bp	39%	29%	39%	48%	39%	7.4	0.06

[a]The Chi square statistic shows the P-value for the association between the treatment used and the occurrence of the more common amino-acid alterations and promoter inserts in *Zymospetoria tritici* isolates
[b]The site.treatment interaction highlights the amino-acid alterations/promoter inserts where the Chi square results for the associations with treatments differed significantly between locations (Table 3.4)
[c]Del = ΔY459/G460

High levels of genotypic diversity were seen in the four treatment groups (Table 3.9), but were highest in epoxiconazole treated and lowest in the pre-treatment groups. Rarefaction analysis showed that if sample sizes were equal, diversity between treatments would have been similar (Table 3.9). Many of the *CYP51* variants were shared between treatments,

although both the epoxiconazole and metconazole collection had almost double the number of unique *CYP51* variants compared to the mixture collection (Table 3.10). Population differentiation between treatment groups was low, and ranged between G_{st} of 0.001 and 0.02 (Table 3.11).

Table 3.9 Summary statistics of genetic diversity found at the *CYP51* gene in *Zymoseptoria tritici* isolates collected from plots treated with epoxiconazole, metconazole, both together (mixture) and from a pre-treatment collection

Treatment	N	S	h	Hd	*SS*	*NSS**	*Var*	*RareFn*	*NSS/SS*
Pre-treatment	96	42	20	0.802	20	17 Del	18	17	0.85
Epoxiconazole	95	44	33	0.907	25	16 Del	28	28	0.64
Mixture	106	41	30	0.916	21	15 Del	25	23	0.71
Metconazole	96	37	23	0.888	19	15 Del	22	21	0.78

N = sample size
S = number of segregating (polymorphic) sites (incl 6 bp deletion)
h = number of nucleotide haplotypes
Hd = haplotype diversity
SS = number of synonymous substitutions
NSS * = number of non-synonymous substitutions and deletion
Var = actual number of *CYP51* variants
RareFn: Potential numbers of *CYP51* variants if all sample sizes were equal to the smallest current sample size

Table 3.10 Proportion of common haplotypes between treatments, and unique haplotypes within treatments (in bold numbers)

Treatment	Pre-treatment	Epoxiconazole	Mixture	Metconazole
Pre-treatment	**0.22**			
Epoxiconazole	0.72	**0.32**		
Mixture	0.72	0.57	**0.16**	
Metconazole	0.44	0.43	0.52	**0.32**

Table 3.11 Pairwise comparisons population differentiation (G_{st}) in the *Zymoseptoria tritici* populations from each of the four treatments (averaged over all locations)

Treatment	Pre-treatment	Epoxiconazole	Mixture	Metconazole
Pre-treatment	*			
Epoxiconazole	0.02	*		
Mixture	0.02	0.005	*	
Metconazole	0.01	0.01	0.001	*

3.3.5 Effect of individual amino-acid alterations on sensitivity

A general linear regression model was used to extract significant effects of alterations and interactions on epoxcionazole and metconazole sensitivity (Table 3.12). Changes at the V136 position, the I381V position, a deletion at Y459/G460 and an insert in the *CYP51* promoter region all decreased epoxiconazole sensitivity. S188N interacted with I381V to decrease sensitivity to epoxiconazole. Conversely, an increase in sensitivity to epoxiconazole was noted if S188N was present with A379G, and in isolates where an insert and a rare alteration were combined. For metconazole sensitivity, the S188N mutation, ΔY459/G460 and an insert in the promoter region, in addition to the interaction between S524T and I381V, all decreased sensitivity. The S188N mutation also interacted with A379G to increase sensitivity to metconazole. As no individual amino-acid alterations were found in isolation in the current data set, combinations of amino-acid alterations (*CYP51* variants) are the focus for the remainder of the study.

Table 3.12 Effects of *CYP51* amino-acid alterations, and promoter insertions on (top) epoxiconazole sensitivity, and (bottom) metconazole sensitivity

	estimate	s.e.	t pr.
Constant	-1.57	0.18	<0.001
S188N 1	-0.47	0.28	0.1
V136 1	0.75	0.18	<0.001
A379G 1	0.24	0.16	0.15
*S188N 1 .A379G 1**	*-0.93*	*0.18*	*<0.001*
I381V 1	0.44	0.18	0.01
S188N 1 .I381V 1	*1.13*	*0.28*	*<0.001*
S524T 1 .I381V 1	*0.26*	*0.18*	*0.15*
I381V 0 .D134G 1	*1.214*	*0.21*	*<0.001*
I381V 1 .D134G 1	*-0.116*	*0.17*	*0.5*
Y461H 1	-0.062	0.08	0.4
Del 1	0.322	0.09	<0.001
S524T 1	0.26	0.19	0.2
S524T 0 .Rare 1	*0.72*	*0.15*	*<0.001*
S524T 1 .Rare 1	*-0.304*	*0.2*	*0.1*
V136 1 .S524T 1	*0.123*	*0.19*	*0.5*
In 1	0.39	0.07	<0.001
In 1 .Rare 1	*-0.64*	*0.22*	*0.004*

	estimate	s.e.	t pr.
Constant	-1.05	0.08	<0.001
S188N 1	0.50	0.10	<0.001
S188N 1 .A379G 1	*-0.61*	*0.18*	*<0.001*
I381V 1	-0.03	0.10	0.768
I381V 0 .D134G 1	*0.66*	*0.23*	*0.005*
I381V 1 .D134G 1	*-0.01*	*0.09*	*0.893*
S524T 1	0.1	0.09	0.272
S524T 1 .I381V 1	*0.35*	*0.11*	*0.001*
A379G 1	0.31	0.17	0.075
Del 1	0.27	0.10	0.004
In 1	0.27	0.06	<0.001

Rare = group of mutations seen less than 5 times
In = any insert in the promoter region
Del = double deletion (ΔY459/G460) in the *CYP51* gene
Numbers after factor names: 0 represents the absence of that alteration, and 1 represents the presence of that alteration
*Interactions between individual factors are in italics

3.3.6 Effect of treatment on frequencies of *CYP51* variants

In comparison to the pre-treatment collection, changes in frequency of *CYP51* variants after treatments were apparent but not always statistically significant (Table 3.13). Significant changes in frequency were seen in; *CYP51* variant 11, where numbers were reduced after all treatments; *CYP51* variant 25 where numbers increased after the metconazole and mixture treatments but remained unchanged after epoxiconazole: *CYP51* variant 31, where a significant increase was observed after the epoxiconazole treatment; and *CYP51* variant 3, where a reduction in frequency was seen after the epoxiconazole and mixture treatments. *CYP51* Variant 31 without the ~800 bp insert (31a) was seen much more frequently after epoxiconazole treatment and *CYP51* variant 4 with that insert (4b) was found significantly less frequently after the mixture treatment (Table 3.13).

Table 3.13 Logistic regression results comparing the relationship between treatment and the frequency of the more frequent (n > 10) *CYP51* variants

		Frequency of *CYP51* variant [a]								Insert [b]				
Treatment	n	3	4	11	22	25	31	32	35	4b	31a	31b	35a	35b
Control	96	8.1	7.1	39.1	8.1	3.1	3.1	2.1	13.1	7.1	3.1	0.1	5.1	8.1
Epox	95	1.1*	2.1	20.1**	11.1	2.1	20.1***	8.1	6.1	1.1	13.1*	7.1	2.1	4.1
Mixture	106	1.1*	2.1	18.1***	11.1	16.1**	11.1	4.1	13.1	1.1*	3.1	8.1	5.1	8.1
Met	97	6.1	2.1	20.1**	3.1	13.1*	7.1	2.1	20.1	2.1	4.1	3.1	7.1	13.1
Chi-sq pr.		0.01	0.155	<0.001	0.1	<0.001	<0.001	0.1	0.03	0.04	0.008	0.008	0.4	0.15

[a] In order to be able to include an analysis of the *CYP51* variants with and without the ~800 bp insert (one *CYP51* variant was not observed in the pre-treatment collection) data were transformed by adding 0.1

[b] *CYP51* variants which had individual isolates with and without the ~800 bp insert in large enough numbers to allow statistical analysis

n = total sample size per treatment. Control = pre-treatment collection

* represents the level of contribution of each treatment to the relevant Chi-sq result. * Significant to P = 0.05; ** Significant to P = 0.01 ;*** Significant to P < 0.001

3.3.7 Evolutionary history of the whole collection

Intragenic recombination in *Z. tritici* populations is a distinct possibility (Brunner *et al*., 2008). The presence of recombination will bias the reconstructed ML tree (Arenas & Posada, 2010), hence evidence of potential recombination was sought. The median-joining network (Figure 3.5) highlighted numerous reticulations, which are evidence of homoplasy due to either independent mutation events or intragenic recombination (Brunner *et al*., 2008).

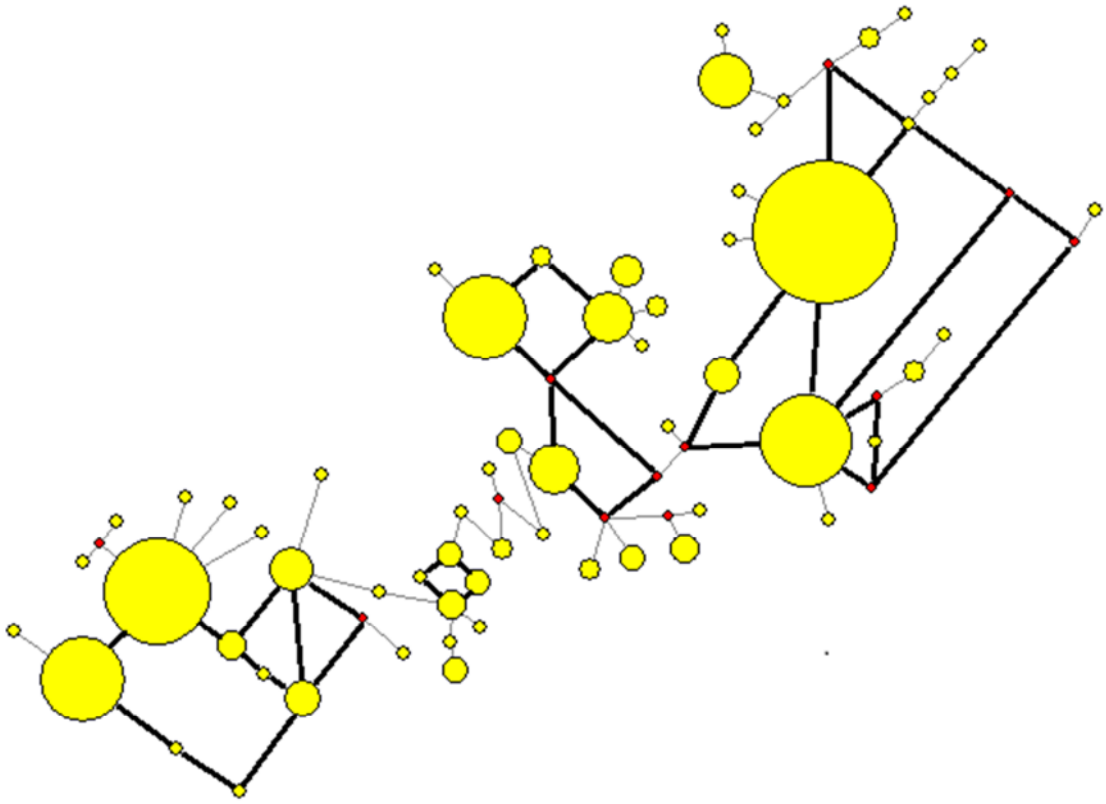


Figure 3.5 Phylogenetic relationship between 61 (+ ST1) haplotypes in a median-joining network used to highlight the high number of reticulations (thick lines) in the population. Yellow circles each represent a single nucleotide haplotype, and circle size is proportional to number of isolates with that haplotype. Red circles represent median vectors (hypothetical haplotypes)

The GARD analysis identified one recombination break point (RBP) in the whole data set (KH-test $P < 0.001$), at nucleotide position 1141 (after introns were removed) (Figure 3.6).

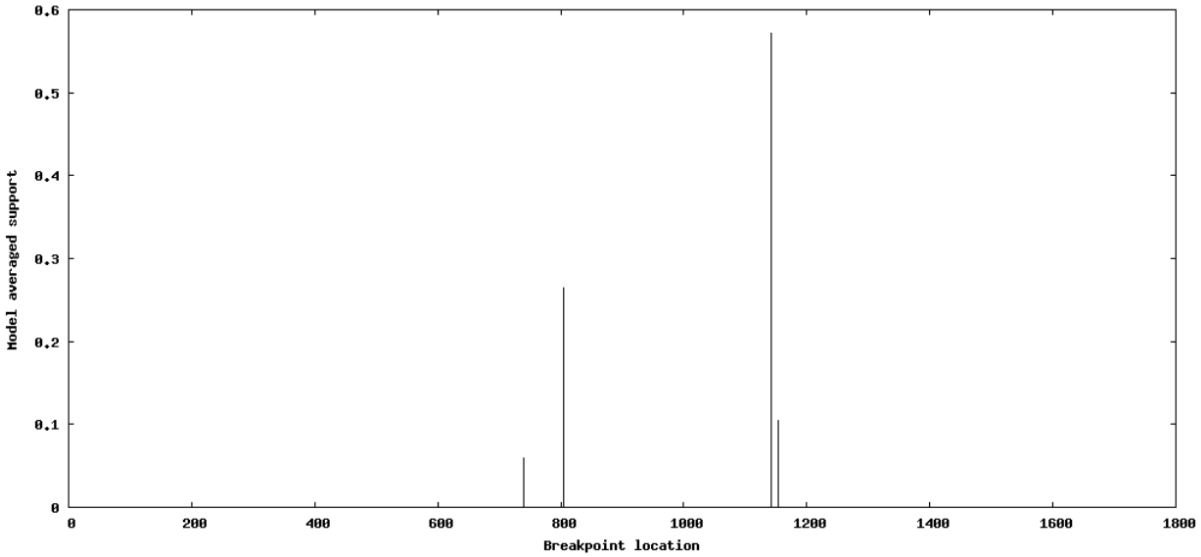


Figure 3.6 Identification of intragenic recombination break points (RBP) in the *CYP51* gene of *Zymoseptoria tritici*. A single RBP at 1141 bp had a significant KH-test $P < 0.001$

The phylogenetic reconstruction of sequence data from the left and from the right of the RBP showed that the topology from the left of the RBP was very similar to the topology from the original data set. However, the topology from the right of the RBP looked different (Figure 3.7), and ten *CYP51* variants (16%) which moved between clades were highlighted. These *CYP51* variants were identified as potential recombinants, and amounted to 16 isolates in total.

Figure 3.7 Reconstruction of phylogenetic trees. Branches with bootstrap values of 70 or greater are labelled. Far left: the original data set, prior to the removal of recombinants. Middle: data from the left of the recombination break point. Right: data from the right of the recombination break point

After the potential recombinant isolates/variants were removed from the data set, a new maximum-likelihood tree was constructed (Figure 3.8). This tree also showed two distinct groups, which were supported by 100% bootstrap value. *CYP51* variants possessing most of the more common amino-acid alterations, L50S, I381V, V136A/C, S524T, Y461S, and A379G, were present in both clades. Some alterations were found only in one clade; Clade A: D134G, V136G, A311G, I377V, Y459S/D, and V490L; Clade B: S188N, N513K and ΔY459/G460, N284H, I384M and A410S. The amino-acid mutation S188N was common among all *CYP51* variants in Clade B. *CYP51* variants with the 120 bp insert also fell into Clade B only, but the ~800 bp insert was found in both clades (Figure 3.8).

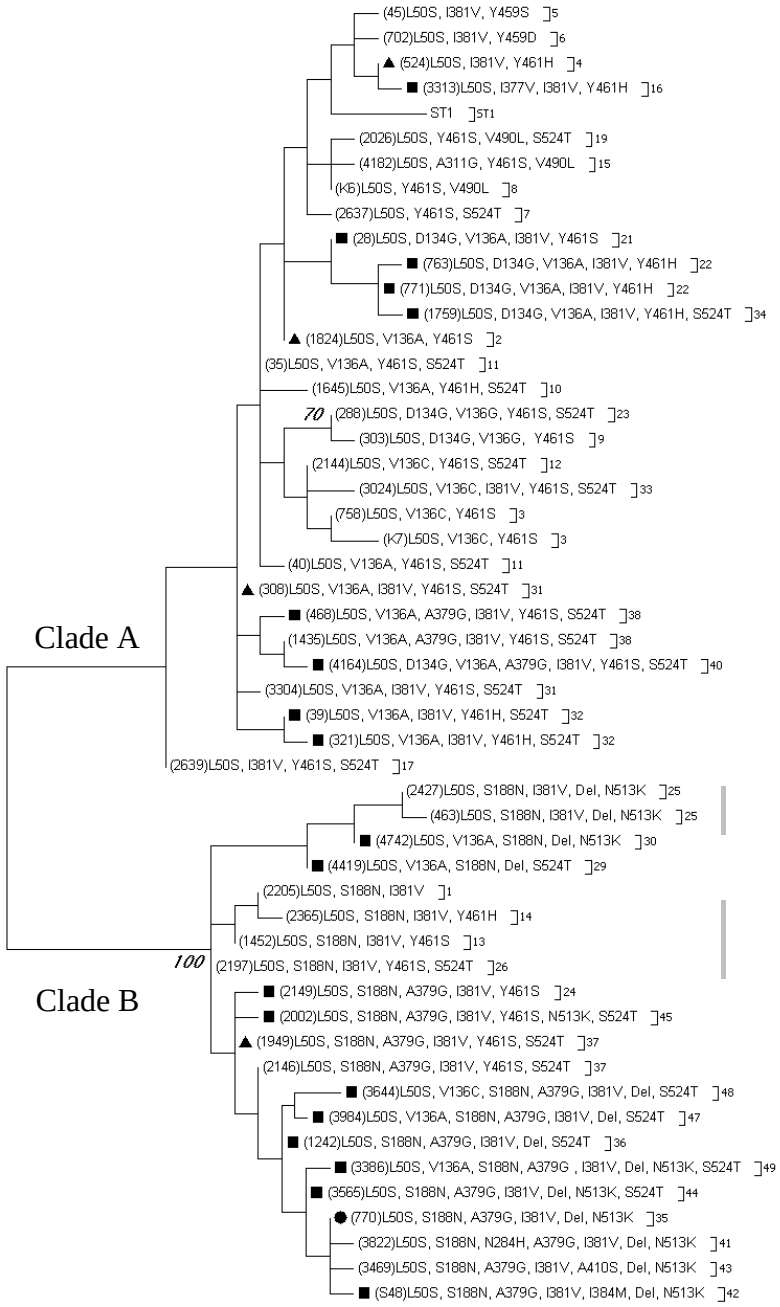


Figure 3.8 After potential recombinants were removed: un-rooted phylogenetic analysis using Maximum Likelihood method. The tree with the highest log likelihood (-2963.8) is shown. The analysis involved 52 nucleotide haplotypes, which included sequence data from the wild-type ST1. There were a total of 1635 positions in the final dataset. Branches with bootstrap values of 70 or greater are labelled. Branch labels: in parenthesis the nucleotide haplotype name; the individual *CYP51* alterations in each *CYP51* variant; outside the square bracket is the *CYP51* variant name as in Table 3.3 (some *CYP51* variants are present in more than one nucleotide haplotype). The symbol at the beginning of branch labels represents the presence or absence of the ~800 bp insert in the nucleotide haplotype: no symbol = no insert; triangle = some isolates have and some isolates have no insert; square = all isolates have the insert; circle = all except one has the insert, and the final isolate has the ~300 bp insert. Grey bars to the right cover branches where the 120 bp insert is present; *CYP51* variants 25, 1, 13, 14 and 26

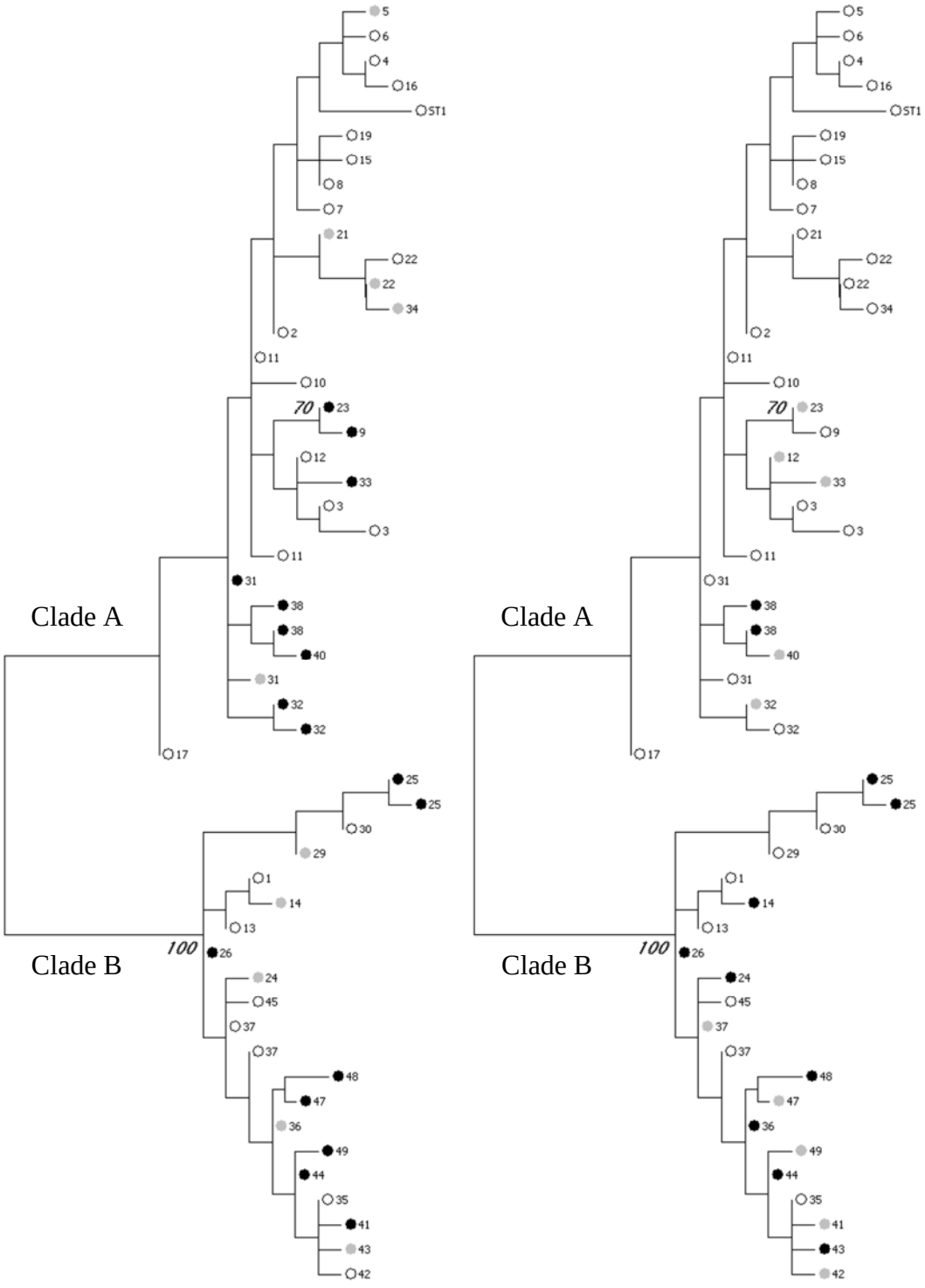


Figure 3.9 Phylogenetic tree, excluding recombinants, highlighting epoxiconazole and metconazole sensitivity ($log_{10}EC_{50}$ mg L^{-1}) groups. Epoxiconazole sensitivity (left): white spots = < -0.3; black spot = > 0.176, grey spot = between -0.3 and 0.176. Metconazole sensitivity (right) white spots = < -0.523; black spot = > 0.0, grey spot = between -0.523 and 0.0

3.4 Discussion

In the populations analysed here, wild-type strains were absent, and the population was very diverse. In total, 49 different *CYP51* variants, 55 when the inserts in the promoter region are included, were identified. V136A and Y461S were still the most commonly found alterations in populations prior to treatment, although both have declined in frequency since the study done by Kildea (2009), and V136A + Y461S + S524T is still the most common *CYP51* variant as found by Kildea (2009). A substantial increase in the frequency of the I381V and S524T mutations has taken place since the work done by Kildea (2009), and current levels of the V136A + I381V combination are higher than those shown in Kildea *et al*. (2014). As well as two new mutations and the many *CYP51* variants seen, ten of which have accumulated seven or more *CYP51* alterations, the current populations also contained three different size inserts in the promoter, one which has not been reported previously.

Previous workers have demonstrated the effects of individual amino-acid alterations on sensitivity and protein function using heterologous expression (Cools *et al*., 2010, Cools *et al*., 2011), and on protein structure using molecular modelling (Mullins *et al*., 2011). Similar studies were beyond the scope of this work, but using regression analysis, a number of amino-acid alterations and some combinations of alterations which affected epoxiconazole and metconazole sensitivity were identified. As demonstrated in previous studies (Leroux *et al*., 2007, Cools *et al*., 2010, Cools *et al*., 2011, Leroux & Walker, 2011), some combinations of amino-acid alterations, with or without other mechanisms, had greater effects on azole sensitivity than others. Thus, the effect of each *CYP51* variant on sensitivity to different azoles depends on the details of the variant, i.e. an additional mutation may make a *CYP51* variant more or less sensitive depending on the other mutations in that variant.

A general decrease in sensitivity was associated with an increase in the number of alterations in the *CYP51* variants, as has been shown previously (Leroux *et al.*, 2007). Isolates from two of the more frequent *CYP51* variants which had only accumulated three alterations (variants 3 and 4) were mostly sensitive (Figure 3.3) to both epoxiconazole and metconazole. The most common *CYP51* variant in this collection (variant 11) was, on average, sensitive to both epoxiconazole and metconazole, and arose from the addition of S524T to the even more sensitive variant 2, L50S, V136A and Y461S. The further accumulation of alterations to this *CYP51* variant, first I381V (variant 31) which has previously been reported (Cools *et al*., 2011, Leroux & Walker, 2011, Buitrago *et al*., 2014), then A379G (variant 38) and finally D134G (variant 40), had the effect of reducing sensitivity to both fungicides, although having more of an impact on epoxiconazole.

Even though I381V has been associated with the increase in strains with reduced azole sensitivity in recent years (Cools *et al*., 2010), it was present here in many *CYP51* variants (~ 70%) spread throughout the range of phenotypes, sensitive as well as less sensitive. On its own, I381V is deleterious, but an alteration at the 459-461 coding positions partially restores the protein function (Cools *et al*., 2010). Leroux *et al*. (2007) found that TriR6 variants, I381V + mutation at 459-461, predominant in Europe since monitoring began (Stammler *et al*., 2008, Stammler & Semar, 2011), were more sensitive to metconazole than epoxiconazole, but the TriR7 variants, I381V + ΔY459/G460, affected epoxiconazole and metconazole sensitivity in a similar manner. In contrast, isolates studied by Cools *et al*. (2012), which had I381V + ΔY459/G460 plus S188N and N513K, were selected for more by metconazole than by epoxiconazole. In Ireland, I381V + alteration at 459-461 has been less prevalent (Stammler & Semar, 2011) until recently, and in this work has been mostly found in combination with other alterations, which have varying effects on fungicides (Table 3.3).

This change in frequency of particular *CYP51* alterations probably reflects changes in fungicide usage patterns over time.

CYP51 variants which had both the V136A and I381V alterations were generally less sensitive to epoxiconazole than metconazole. Previously, strains with the V136A alteration have been shown to remain sensitive to metconazole (Kildea, 2009) and tebuconazole (Cools *et al*., 2011), while sensitivity to epoxiconazole and other azoles such as prothioconazole and prochloraz tend to decrease in its presence (Cools *et al*., 2011). Hence, it is possible that the occurrence of the V136A alteration creates a barrier to reduced metconazole sensitivity in these and other *CYP51* variants: either, isolates with the V136A mutation are sensitive to metconazole and so it works even though these *CYP51* variants are common, or it is possible that further changes are necessary for V136A to be able to affect the metconazole binding position in order to have an effect on sensitivity. Indeed, in this collection, the addition of S524T to that combination tended to decrease sensitivity to both fungicides, in agreement with Cools *et al*. (2011) who found that S524T reduced sensitivity to all azoles tested (they did not include metconazole). Even so, amongst the eight *CYP51* variants with those three alterations, only a single *CYP51* variant, L50S, V136A, A379G, I381V, Y461S, S524T (variant 38) was metconazole insensitive. It is possible that the A379G mutation, which was always found with I381V in this collection, and which was selected strongly by the metconazole and mixture treatments, reduces sensitivity to metconazole even in the presence of V136A.

CYP51 variants with an insert in the putative *CYP51* promoter had varying effects on azole sensitivity (Table 3.3 and Figure 3.3), but in *CYP51* variants which were found frequently (n > 10), the presence of either the 120 bp or ~800 bp inserts reduced sensitivity to both fungicides, in agreement with Cools *et al*. (2012), Chassot *et al*. (2008) and Buitrago *et al*. (2014), but conflicting with Kildea (2009) and Leroux and Walker (2011). Cools *et al*. (2012) found an association between a 120 bp insert in the promoter and *CYP51* over-

expression in *Z. tritici* isolates with reduced sensitivity. Prior to that finding, over-expression of the target gene had been shown to contribute to reduced DMI sensitivity in numerous other plant pathogens: *Ventura inaequalis* (Schnabel & Jones, 2001), *Penecillium digitatum* (Hamamoto *et al*., 2000), *Blumeriella jaapii* (Ma *et al*., 2006), *Cercospora beticola* (Nikou *et al*., 2009) and *Monilinia fucticola* (Luo & Schnabel, 2008). While Chassot *et al*. (2008) inferred that a large insert of ~1000 bp was associated with over-expression of the *Z. tritici CYP51* gene, Leroux and Walker (2011) did not make the same connection. Further analysis of the putative *CYP51* promoter region is needed to confirm if these large inserts are associated with *CYP51* over-expression.

The population prior to fungicide application, from which selection could take place, was genetically very diverse, with many amino-acid alterations and *CYP51* variants present. In comparison to the pre-treatment collection, solo fungicide treatments were seen to select specific mutations, a feature of azole fungicides which has been highlighted previously. But whilst solo epoxiconazole and metconazole selected the D134G, V136A, S188N, A379G, Y461H, ΔY459/G460, N513K and S524T alterations differentially, selection for these alterations by the mixture was mostly positive, and neutral in one case. In addition, the frequency of I381V, which was positively selected by all fungicides, almost doubled after the mixture. In the same way, *CYP51* variants which were selected differentially by the solo epoxiconazole and metconazole treatments were also mostly positively selected by the mixture. The observed selection for amino-acid alterations/variants was in agreement with sensitivity data for all three fungicide treatments, as seen in Chapter 2. In that chapter, when EC_{50} values were compared, cross-resistance between the epoxiconazole applied alone and the metconazole applied alone was observed, but not complete. However, the mixture reduced sensitivity to both fungicides. When mixing two fungicides as an anti-resistance tactic, it is expected that each component of the mixture would negatively select isolates which have

been positively selected by the other component, therefore reducing final selection by each component and maintaining sensitivity. In this case, the mixture positively selected most *CYP51* variants and little negative selection took place, indicating that this specific mixture of metconazole and epoxiconazole increases the selection for *CYP51* variants with reduced azole sensitivity.

A single alteration can affect the protein in different ways. It may have little direct effect on the function of the protein, it might be a direct mutation of a key residue or it could change the structure of the protein which moves a residue out of reach of an azole (Mullins *et al*., 2011). When an amino-acid change does affect protein function it can result in a reduced azole binding affinity or a loss of azole accommodation in the active site (Mullins *et al*., 2011). Multiple alterations together may either further reduce sensitivity or may act in a compensatory capacity. Two of the amino-acid alterations known to affect azole sensitivity, V136A and I381V, have been shown to be deleterious when found in isolation; only when they arose in isolates which already had other specific alterations could they affect sensitivity (Cools *et al*., 2010, Cools *et al*., 2011). Those other alterations, in this case, a mutation or deletion at 459-461 coding positions, could be considered to be compensatory, i.e. they restore or improve the function of *CYP51* variants which otherwise would have low fitness (Mullins *et al*., 2011). Indeed, changes at the 459-461 positions appear to be essential for other alterations which reduce azole sensitivity (Buitrago *et al*., 2014). In a similar manner, the absence of a *CYP51* variant with the V136A and I381V alterations together in earlier populations may have been due to structural constraints in the protein which would have limited the accumulation of those alterations (Cools & Fraaije, 2008). If this were the case, compensatory mutations may have had a role to play in the appearance of this, and possibly other combinations.

The S524T alteration, identified retrospectively in isolates from as early as 2001 and in combination with the now rare Y137F (Cools *et al*., 2011), re-emerged in isolates from 2006 (Kildea *et al*., 2014), and is presently found in many *CYP51* variants spread throughout Ireland and Europe (Buitrago *et al*., 2014, Kildea *et al*., 2014). Cools *et al*. (2011) showed that in a *CYP51* variant with L50S, D134G, V136A and Y461S, the S524T mutation had the effect of opening up the binding cavity, which removed residues from the immediate vicinity of the epoxiconazole bound ligand, which led to reduced azole binding and loss of efficacy. Mullins *et al*. (2011) propose that this is a feasible evolutionary solution to azole inhibition; accommodating the larger azole molecules but reducing their activity, while restricting the structural rearrangements to maintain the integrity of the enzyme. They also showed that this alteration reduced sensitivity to all fungicides tested. It is possible that the S524T mutation may have facilitated the continued accumulation of alterations, i.e. acted as a compensatory mutation.

In some *CYP51* variants, e.g. variants 31 and 35, where a broad range in sensitivity was observed, differences in the putative promoter region (the presence of a ~800 bp insert) is likely to have contributed to these differences in sensitivity. However, in *CYP51* variants where no inserts in the promoter were identified, e.g. variant 11, where EC_{50} values of individual isolates ranged from -2.2 to 0.74 ($\log_{10}$ mg L^{-1}), alternative mechanisms may be acting. Over-expression of drug efflux proteins is a possibility, and is the most frequently documented mechanism for DMI resistance in *Candida albicans* (Leroux & Walker, 2011). While Cools and Fraaije (2013) confirm that the potential for this mechanism to affect azole resistance is there, over-expression of drug efflux proteins ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters have not been shown specifically as a DMI resistance mechanism in *Z. tritici*. Leroux and Walker (2011) however, suggested that this mechanism may be involved in observed multi-drug-resistant strains, and in a more recently

paper Fillinger *et al*. (2014) demonstrated that active efflux, probably caused by over-expression of the membrane transporter gene *MgMFS1*, mediated multi-drug-resistance (resistance to DMIs and SDHIs).

The high level of genetic diversity currently seen in the *Z. tritici CYP51* gene in Ireland is comparable to populations from other countries (Schnieder *et al*., 2001, Brunner *et al*., 2008, Boukef *et al*., 2012, Drabešová *et al*., 2013, Estep *et al*., 2014). But how is this diversity generated and dispersed? Mutations are the ultimate source of genetic diversity (McDonald, 2004); when a mutation has a fitness benefit in the presence of a fungicide, it can survive and reproduce, and under fungicide selection will eventually increase as a proportion of the whole population. Knowledge of whether the specific mutations which confer a decrease in azole sensitivity have emerged once and have been dispersed to different geographic regions, or have emerged multiple times in different genetic backgrounds (homoplasy) is important for understanding disease epidemiology and the evolution of resistance (Brunner *et al*., 2008). Brunner *et al*. (2008) proposed that the multiple emergence of a mutation into different genetic backgrounds is not likely in *Z. tritici* populations, given the low likelihood of all alterations and combinations of alterations arising independently in different regions. The high level of gene flow observed in this study, which is comparable to other studies (Linde *et al*., 2002, Zhan *et al*., 2003, Zhan & McDonald, 2004), would certainly facilitate the dispersal of an emerged *CYP51* variant, and the current phylogenetic analysis appears to provide evidence for such events. For example, S188N, N513K, ΔY459/G460 and the 120 bp insert are only seen in clade B, demonstrating a common ancestral lineage. Even so, extensive homoplasy was also observed, as evidenced by the multiple recurrences of the V136A, I381V, Y461S and S524T alterations throughout the phylogeny. Brunner *et al*. (2008) also found evidence of homoplasy, and in particular demonstrated that the I381V mutation arose twice in different ancestral lineages. Identical alterations which are found in

different genetic backgrounds occur either through *de novo* (new) mutation events or through intragenic recombination events. Brunner *et al*. (2008) made a strong case for the role of intragenic recombination in the introduction of novel alleles into new genetic backgrounds, which they feel contributed to the rapid evolution of *Z. tritici* populations. Intragenic recombination was identified in the current study, but considering the high level of genetic diversity, the number of potential recombinants found was relatively low. Only 16% of the overall haplotype diversity were recombinants, which is comparable to the older European populations before many of the mutations in the recent populations had in fact emerged (Brunner *et al*., 2008). In order to be able to construct an undistorted evolutionary history of the population (Schierup & Hein, 2000, Graham *et al*., 2005, Lemey & Posada, 2009), these potential recombinants were removed from the analysis, after which there was still a considerable level of homoplasy in the phylogeny. While it is possible that other intragenic recombination events occurred but were not detected in this analysis, *de novo* mutations in different genetic backgrounds are the most likely explanation for this high level of homoplasy.

The split of this population into two clades has been previously observed by Leroux *et al*. (2007) and Brunner *et al*. (2008), both of whom found one clade to be less sensitive to azoles than the other. The current analysis demonstrates a somewhat similar result (Figure 3.9), with the more sensitive *CYP51* variants found in Clade A, but seems to affect metconazole more than epoxiconazole. Leroux *et al*. (2007) propose that these two clades are separate genetic units, of which one is more naturally insensitive to DMIs. Further analysis is needed to confirm the function of the two separate clades, and whether the clades in the current work are related to those demonstrated previously.

In a recent review by Cools *et al*. (2013), the authors propose that the fitness costs and trade-offs associated with azole resistance will mean that this group of fungicides is not likely

to succumb to complete field resistance in the near future. It has been hypothesised that there are likely to be constraints on the number of alterations that the *CYP51* gene can tolerate while maintaining its basic functions (Mullins *et al*., 2011), but as we have seen in the recent *Z. tritici* populations, for example with the V136A and I381V combination, compensatory mechanisms could allow for the continued accrual of alterations, and until we see the combinations, we do not know if they will happen. Even if there are constraints on the accumulation of *CYP51* alterations, the presence of other mechanisms which reduce azole sensitivity, such as over-expression of the *CYP51* gene, may fill that evolutionary gap. The 120 bp insert has only been reported in *CYP51* variants with five or less amino-acid alterations. Considering that it looks as though the number of amino-acid alterations in a *CYP51* variant has an effect on sensitivity, it would be of some concern if this 120 bp insert were to recombine with some of the more evolved/insensitive *CYP51* variants, especially if insensitive variants which are also over-expressed have the potential to affect field efficacy of all azoles (Cools *et al*., 2013). Additionally, the potential for over-expression of drug efflux proteins to reduce azole sensitivity, although probably limited in practice (Cools *et al*., 2013), could add to the ability of *Z. tritici* populations to respond to selection.

The increased amount of *CYP51* variation in the current study compared to previous studies demonstrates the continued evolution of the *CYP51* gene. This evolution has been driven by the continued use of azoles in general, but the results here suggest that using mixtures of two azoles speeds up the process. Particularly, as the mixture seemed to select for isolates with reduced sensitivity to both components. This is concerning as azole mixtures are commonly used in winter wheat production and highlights the need to always add a fungicide with a different mode-of-action when using azole mixtures. The complexity of the target protein and fitness landscape (where populations are located on a landscape depending on the genetic distance from each other (Kaplan, 2008)) of *CYP51* variants has been demonstrated in

this chapter and it has become evident that tracking single *CYP51* variants will not suffice to predict field control, or more precisely, field control failure. We have seen that routes around blocked pathways in an evolutionary landscape often exist and that regulatory sequences, other than those already known, can profoundly affect phenotype. For example, the addition of A379G to the V136A mutation may have restricted access of metconazole to the target, reducing the ability of metconazole to work properly. On top of this, the probability that selected codon changes have repeatedly evolved in different genetic backgrounds, and the effect of intragenic recombination on accumulating resistance mechanisms; all of these factors together are likely to ensure the continued evolution of azole insensitivity.

Chapter 4: Succinate dehydrogenase inhibitor fungicides and their effect on baseline and treated *Zymoseptoria tritici* field isolates

4.1 Introduction

SDHIs are single-site inhibitors, like the azoles. The SDHI target site, succinate dehydrogenase, is composed of four subunits, *SdhA, SdhB, SdhC* and *SdhD,* (Stammler *et al*., 2011) of which the *SdhB, SdhC* and *SdhD* directly interact with the SDHI fungicides. They inhibit the succinate dehydrogenase enzyme, which in turn disrupts the tricarboxylic acid cycle (Scalliet *et al*., 2012), which is a key part of aerobic respiration. Resistance to SDHI fungicides were initially thought to be conferred by mutations at one locus (Leroux *et al.*, 2011). That is, full resistance is possibly due to a single amino-acid change in the target protein which does not need compensatory mutations to be fit enough to survive in the field. Lab resistance (the level of reduction in sensitivity varied depending on the mutations) to the different SDHIs has readily developed in mutagenesis studies (Skinner *et al*., 1998, Stammler *et al*., 2011, Fraaije *et al*., 2012, Scalliet *et al*., 2012), demonstrating the potential for SDHI resistance in this pathogen. And so some researchers therefore considered *Z. tritici* to be a high risk of developing resistance (Fraaije *et al.*, 2012). The aforementioned mutagenesis studies all identified, amongst others, the histidine residue in *SdhB* at the 267 codon. The *SdhB* subunit is highly conserved between species (Scalliet *et al*., 2011) and homologous positions of the 267 codon in *Z. tritici* have been identified as resistance hotspots in field resistant isolates of other pathogens (Avenot *et al*., 2008, Ishii *et al*., 2011, Veloukas *et al*., 2013). This similarity in the target protein in several diverse pathogens suggests the same change may have similar effects in *Z. tritici* field isolates. Field resistance towards the first generation SDHI carboxin, and to the "oldest" of the newer SDHIs, boscalid, has been seen in many pathogens (Sierotzki & Scalliet, 2013). While a few individual *Z. tritici* field isolates have been found to have a slightly reduced sensitivity (but low resistance factors) to the

SDHIs (Anon, 2013b), one found in France and one in the UK in 2012 which had the *SdhC* mutations T79N and W80S respectively, resistance is not yet (in 2014) evident in field isolates of *Z. tritici*. Two additional mutations found in field isolates, N225H in the *SdhB* subunit and N86S in the *SdhC* subunit, were cited in a subsequent report (Anon, 2015), but not noted in the minutes of the most recent FRAC meeting (Anon, 2014d). The presence of these multiple mutations suggests that a gradual loss of sensitivity in a step-wise manner is also a possibility.

Knowledge of sensitivity levels in unexposed pathogen populations is essential to identify early shifts in sensitivity; allowing timely adjustment of spray programmes and avoiding loss of control in the field (Avenot & Michailides, 2010). Baseline studies can also be used to determine levels of cross-resistance between active ingredients. Knowing if fungicides are cross-resistant allows management plans to include differentially selecting fungicides in programs. Cross-resistance between some SDHIs has been reported in some pathogens but not in others, e.g. Avenot and Michailides (2010) found cross-resistance between boscalid and penthiopyrad in *Alternaria alternata*, but Gudmestad *et al*. (2013) saw no cross-resistance between the same two fungicides in *A. solani*. As SDHI activity (Scalliet *et al*., 2011) and cross-resistance appear to be pathogen dependent, even though the target gene is highly conserved between species, inferences from other studies should not be made for *Z. tritici*.

In order to manage resistance, the ideal strategy may differ depending on the phase of fungicide resistance the population is at (Van den Bosch *et al*., 2011). Currently the Irish *Z. tritici* population is at different stages in the development of azole and SDHI resistance. With azole insensitive *Z. tritici* strains now well established in most of north-western Europe, the evolution of azole insensitivity is firmly in the selection, if not the adjustment phase. Management must therefore focus on slowing the selection for insensitive isolates and where

necessary and possible, altering standard control programmes in order to maintain control. SDHI insensitivity on the other hand has yet to emerge, so delaying the emergence of resistance and monitoring the population to identify early changes are the main priorities.

Mixing fungicides with different modes-of-action is one approach to slowing the rate of resistance evolution. This method exposes the strains resistant to one component to a different mode-of-action, thereby reducing the rate of increase of those strains. Given the potential for superior disease control and the additional benefits of delayed resistance development, mixtures of fungicides are commonly used for STB control. Indeed, because of the high-resistance risk, manufacturer recommendations state that SDHIs must always be used in combination with other modes-of-action, and SDHI/azole mixtures are now commonly used in wheat production. In the past, resistance management usually commenced after the emergence of resistance (van den Bosch *et al*., 2014); hence most studies have focussed on the selection phase and less on the emergence phase. Even so, Hobbelen *et al.*, (2014) concluded that strategies that are most effective at delaying the evolution of fungicide resistance do not differ between the emergence and the selection phase. Empirical (Mavroeidi & Shaw, 2006, Thygesen *et al*., 2009) and modelling studies (Birch & Shaw, 1997, Hobbelen *et al*., 2011b, Hobbelen *et al*., 2013) have looked at the effects of mixing fungicides, manipulating both dose and ingredients, as a way of managing fungicide resistance. However, the effects of mixing two fungicides both at-risk of resistance - such as azoles and SDHIs - are underrepresented in studies on mixtures (Hobbelen *et al*., 2013). Hobbelen *et al*. (2013) predicted that mixing two at-risk fungicides increased the effective lives of both fungicides compared to sequential use and concurrent use on neighbouring fields, particularly so when strains insensitive to both components were at low initial frequencies.

The study reported here was initiated to establish baseline sensitivities and cross resistance patterns to newly commercialised SDHI fungicides, in Irish *Z. tritici* populations.

Selection experiments were undertaken to determine the impact that mixing an azole and a SDHI fungicide has on *Z. tritici* sensitivity to both fungicides. Field trials were conducted at different locations through Ireland. Treatments were applied at both full and half the recommended dose rates and the sensitivity of *Z. tritici* populations were monitored pre- and post-treatment. Additionally, the sequences of the *Sdh* subunits typically involved in resistance were determined in a sub-set of isolates from the baseline and selection experiment collections; isolates were chosen based on their sensitivity to isopyrazam. The findings presented and discussed provide the empirical data required to formulate strategies to prolong the effectiveness of both azole and SDHI fungicides against STB.

4.2 Materials and methods

4.2.1 Origin of isolates

4.2.1.1 Baseline collection

A collection of 209 field isolates from the years 2005-2010 were used to determine the baseline sensitivity to five of the newer SDHI fungicides, isopyrazam, bixafen, fluxapyraxad, penthiopyrad and fluopyram, as well as to boscalid which has been in use since 2005. Isolates came from commercial fields, representing 21 locations in Ireland, and four locations in the UK for comparison. The UK isolates (Courtesy of J. Blake, ADAS) were collected in 2010 only.

4.2.1.2 Selection experiments- trial design, fungicide application and sampling

Field trials were conducted over two growing seasons between 2011 and 2013, at six locations throughout Ireland (Table 4.1). All trials were laid out as complete randomised block designs with four replicate blocks of six fungicide treatments and an un-treated control. Plots were 2.5 m × 10 m with a 30-40 cm path between plots, and disease was allowed to develop naturally. Fungicide treatments consisted of two foliar applications (referred to as T1

at GS 32-37 and T2 at GS 39-53 (Zadoks *et al*., 1974) depending on location) of the fungicides epoxiconazole (Opus®, BASF) and isopyrazam (Zulu®, Syngenta) as solo a.i.s, and together in a pre-formulated mixture (Seguris®, Syngenta), and at full and half the recommended dose rates (Table 4.2). All fungicides were applied in 200 L/ha water using a knapsack sprayer with compressed air. Each location was sampled prior to spraying in order to determine the pre-treatment distribution of fungicide sensitivity. Approximately 50 diseased leaves were sampled at each location in 2012 and approximately 100 per location in 2013. At the sampling time after treatment (six weeks post T2), approximately 40 diseased leaves were taken, regardless of disease severity, at roughly equal distances apart within each plot, and avoiding ends and edges of plots. All leaves were air dried for five days at room temperature and then stored at -20 °C awaiting pathogen isolation.

Table 4.1 Selection experiment: details of site location, year each location was included, date of fungicide applications and growth stage (GS) at which fungicides were applied, date of disease assessment and GS at that time

Location *(Coordinates)*	*Year*	*Cultivar*	*Resistance rating* [a]	*Date of T1*	*GS* [b] *at T1*	*Date of T2*	*GS* [b] *at T2*	*Date of disease assessment*	*GS at disease assessment*
Julienstown (53.679806, -6.309156)	2012	Cordiale	4	3rd May	33	29th May	39	26th June	73
Killeagh (51.940363, -8.026993)	2012	Einstein	5	2nd May	37	23rd May	45	25th June	73
Oak Park 1 (52.863676, -6.914563)	2012	Cordiale	4	4th May	32	6th June	43	28th June	73
Drogheda (53.740894, -6.404514)	2013	Einstein	5	21st May	32	7th June	45	19th July	81
Midleton (51.823312, -8.168507)	2013	Cordiale	4	20th May	32	6th June	55	18th July	83
Oak Park2 (52.863029, -6.915464)	2013	Cordiale	4	22nd May	37	5th June	49	11th July	79

[a] Resistant rating on a scale of 1-9, 1 = susceptible, 9 = resistant (DAFM https://www.agriculture.gov.ie/publications/2013/)
[b] GS Growth stage (Zadoks *et al*., 1974)

Table 4.2 Treatments used in the selection experiment: application pattern, dose rates applied, fungicides used amounts of active ingredient (a.i.) at each treatment time with overall amount of active ingredient per treatment in parenthesis

Application pattern	*Treatment name* [a]	*Dose* [b]	Active ingredient (a.i.) applied[c]		*Litres/ha applied at T1 & T2 (total a.i. applied)* [d]
			T1	*T2*	
Un-Treated	Un-T	0	None	None	N/A (0 g)
Solo a.i.	EE	1	Epoxiconazole	Epoxiconazole	1.5 (249 g)
	II	1	Isopyrazam	Isopyrazam	1 (250 g)
	ee	0.5	Epoxiconazole	Epoxiconazole	0.75 (124.5 g)
	ii	0.5	Isopyrazam	Isopyrazam	0.5 (125 g)
Mixture	EIEI	1	Epoxiconazole & isopyrazam	Epoxiconazole & isopyrazam	1 (180g & 250g)
	eiei	0.5	Epoxiconazole & isopyrazam	Epoxiconazole & isopyrazam	0.5 (90g & 125g)

[a] Abbreviations denote the first and second sprays. Un-T= un-treated control; E or e: epoxiconazole; M or m: metconazole; uppercase: full dose; lowercase: half dose
[b] Application dose at Treatment 1 and Treatment 2; 1 = the full label recommended dose, 0.5 = half the label recommended dose
[c] Epoxiconazole = Opus® (product of BASF), isopyrazam = Zulu® (product of Syngenta), epoxiconazole + isopyrazam = Seguris® (product of Syngenta).
[d] Active ingredient (a.i.) per litre of product; Opus: 83 g/l; Zulu: 125 g/l; Seguris: 90 g/l epoxiconazole + 125 g/l metconazole

4.2.2 Determination of fungicide sensitivity- baseline and selection experiment

4.2.2.1 Isolation of fungal isolates

Isolations were carried out according to Kildea (2009), as in Chapter 2. For the baseline isolates, single-spore isolates were produced; 50 µl of the stock solution was spread over PGA, after three-five days a single-spore colony was isolated and further grown in Potato Dextrose Broth (PDB) (Sigma-Aldrich Co.) for five-seven days, after which 50 µl of the PDB inoculum was sub-cultured onto antibiotic amended PGA (as above), sealed and incubated at 18 °C for a further three days. Clean cultures were scraped from the plates and individually stored in 30% glycerol at -80 °C until further use.

4.2.2.2 In vitro sensitivity testing- baseline and selection experiment

The sensitivity of all experimental isolates to epoxiconazole and isopyrazam and of all baseline isolates to bixafen, boscalid, fluopyram, fluxapyroxad, isopyrazam and penthiopyrad was determined using a microtitre plate assay as described by Kildea (2009). Initially, SDHI fungicides were dissolved in dimethyl sulfoxide (DMSO), whereas epoxiconazole was dissolved in methanol. All fungicides were subsequently diluted in 100% methanol and added to PDB to give final test concentrations, as in Chapter 2. The remainder of this process was carried out as in Chapter 2.

4.2.3 Molecular study of the *Sdh* genes in isolates from the baseline collection and selection experiment

4.2.3.1 Choosing isolates

A subset of 96 isolates was chosen from the baseline collection for further molecular studies. Isolates were chosen based on their sensitivity to isopyrazam. From each of the five years, 19 isolates were chosen, six of which had low, seven had medium and six had high EC_{50} values

relative to that year's collection. A subset of 96 isolates was also chosen from the selection experiment for further molecular studies. These were chosen regardless of treatment and based on their sensitivity to isopyrazam, with all 96 from the least sensitive range.

4.2.3.2 Collection of fungal DNA, PCR and DNA sequencing- baseline and selection experiment isolates

DNA was extracted as described in Chapter 3. Amplification of the three *Sdh* subunit genes (*B*, *C* and *D*) was carried out using PCR (Biometra TProfessional Basic Gradient) in a final volume of 25 µl containing 1U Phusion® High-Fidelity polymerase (New England BioLabs Inc.), 1 X Phusion® buffer, 200 µM deoxynucleoside triphosphate (dNTP), 300 nM of both forward and reverse primers (Table 4.3) and 20 ng of fungal DNA. Amplification conditions were 98 °C for 30 s, followed by 35 cycles at 98 °C for 10 s, 57 °C (*SdhB*), 64 °C (*SdhC*) or 61 °C (*SdhD*) for 30 s, and 72 °C for 15 s with a final DNA extension at 72 °C for 5 min. PCR products were Sanger sequenced by Eurofins MWG Operon (Ebersberg, Germany) using the respective forward primers as the sequencing primer. The resultant sequences were assembled using CLC Genomics Workbench 5.1 (http://www.clcbio.com [Accessed 28-12-14]). Assembled sequences were edited, aligned to the wild-type (WT) *Sdh* genes from IPO323 and analysed using BioEdit version 7.0.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html [Accessed 28-12-14]).

Table 4.3 Primers used in the polymerase chain reaction to amplify *Sdh* genes from *Zymoseptoria tritici* isolates

Name	Target	Sequence
Mgsdhbf1[a]	*SdhB*	ACTCTTCTCACATACCACACA
Mgsdhbr1		CTTTCCAATCATCTCGTTCCAT
Mg-SdhC-F	*SdhC*	CCAGTAAGAGGTCCGATTATTACC
Mg-SdhC-R		ACCGTCAACATTCCGTACTTC
Mg-SdhD-F	*SdhD*	CGGGAATAACCAACCTCACT
Mg-SdhD-R		CCTCACTCCTCCAAACCGTA

[a] *SdhB* primers as per Fraaije *et al.* (2012)

4.2.4 Data analysis

EC_{50} values were calculated and adjusted as in Chapter 2. For the baseline collection, normality of the non-transformed EC_{50} distribution for each fungicide was tested using the Shapiro-Wilk test. Associations between SDHIs were evaluated using Pearson's correlation analysis. For the selection experiment, EC_{50} values were analysed as in Chapter 2. Briefly, data from the pre-treatment sampling time (Pre-T) were analysed using REML, whilst data from the post treatment sampling time were analysed using REML with contrasts (Crawley, 2005), using the FCONTRASTS procedure. In the model, treatment (7 levels) was considered a fixed effect, whilst location (six levels), replicate (four levels) and location.treatment were considered random effects. Contrasts were estimated separately for epoxiconazole and isopyrazam sensitivity. Contrasts 1 and 2 included only data from the full rate treatments. Isolations were not made from the half rate solo isopyrazam or azole/SDHI mixture treatments in 2012, so the dose rate contrast (contrast 3) applied only to 2013 data. Disease severity and yield were analysed as in Chapter 2.

4.3 Results

4.3.1 Baseline fungicide sensitivity distributions

Isolates were tested for their sensitivities to bixafen, fluopyram, fluxapyroxad, isopyrazam, penthiopyrad and boscalid; 2005 n = 26; 2006 n = 36; 2007 n = 19; 2009 n = 80; 2010 n = 48. There was no difference in sensitivity between the UK isolates and Irish isolates from 2010 ($P > 0.05$), so the whole collection was further analysed as a single unit. For all fungicides tested, including boscalid, there was no difference in sensitivity between the years of sampling ($P > 0.05$). The intrinsic sensitivity of the *Z. tritici* collection to the SDHI fungicides varied; ranked in the order of lowest median EC_{50} first, isopyrazam < fluxapyroxad < penthiopyrad < bixafen < boscalid < fluopyram. Boscalid and fluopyram had significantly

higher EC_{50} values than the other four fungicides ($P < 0.001$), and the distribution of their transformed EC_{50} values were non-normal, based on W-test they were leptokurtic and skewed to the right respectively (Table 4.4).

Table 4.4 Summary of baseline sensitivity (EC_{50} mg L^{-1}) of *Zymoseptoria tritici* isolates for each active ingredient (a.i.) tested

Active ingredient	Range	Mean	Median	VF [a]	W test [b]
Bixafen	0.018 - 0.822	0.209	0.165	45	$P = 0.5$
Boscalid	0.066 - 2.903	0.748	0.661	44	$P = 0.02$
Fluopyram	0.076 - 10.95	1.12	0.588	143	$P < 0.001$
Fluxapyroxad	0.022 - 0.668	0.188	0.149	30	$P = 0.34$
Isopyrazam	0.012 - 3.121	0.201	0.146	271	$P = 0.1$
Penthiopyrad	0.015 - 1.618	0.228	0.163	105	$P = 0.17$

[a] variance factor was calculated by dividing the highest EC_{50} in the range by the lowest EC_{50} in the range
[b] W test is the Shapiro-Wilk test for normality of distribution (log scale); $P < 0.05$ is not normally distributed

Pearson's correlation indicated that there was cross-resistance between all fungicides (P < 0.001). Cross-resistance with other SDHIs was weakest for fluopyram (Figure 4.1).

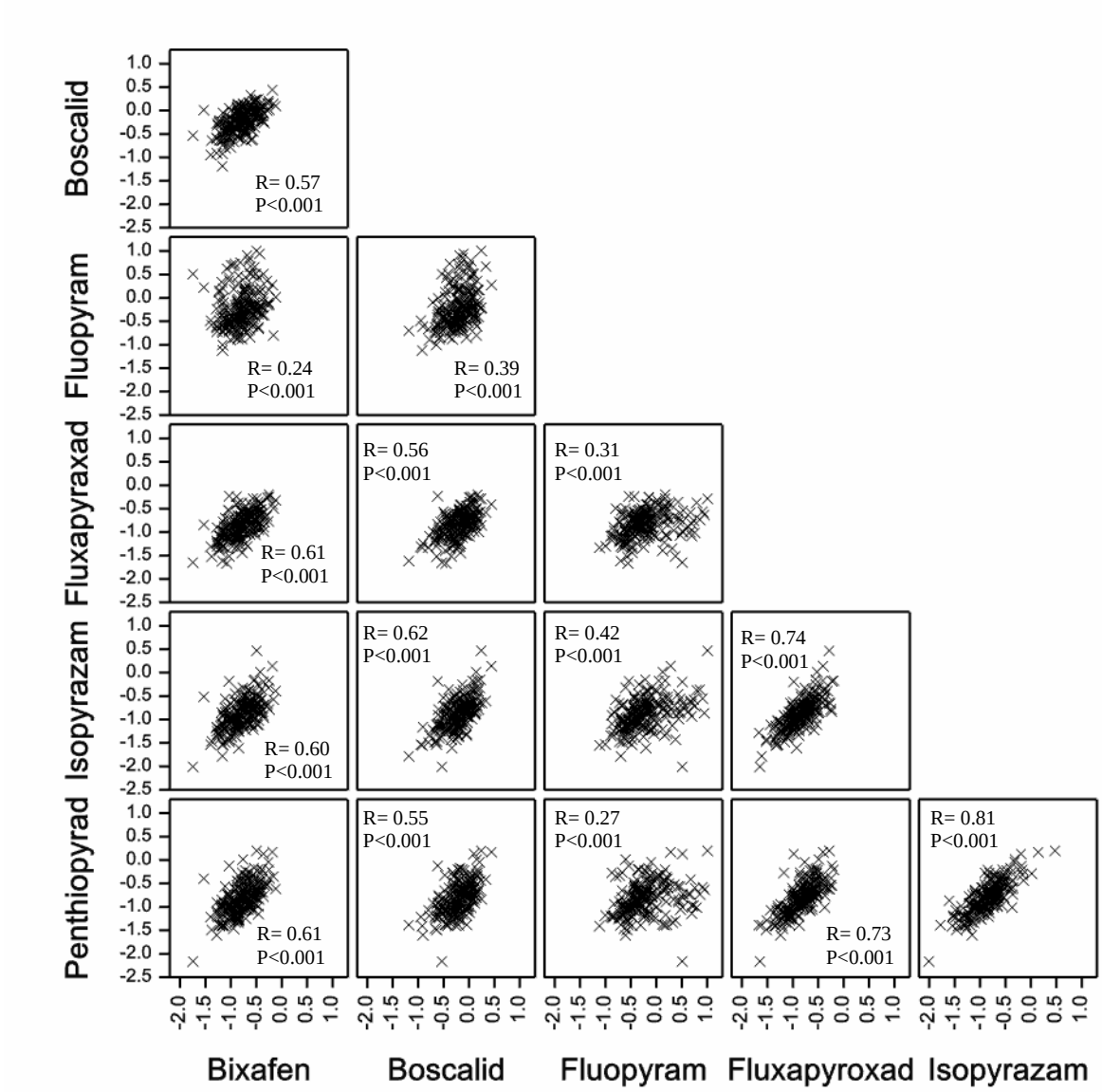


Figure 4.1 Matrix of correlations between fungicide sensitivities for baseline isolates (n = 209). Sensitivity was measured as EC_{50} values (mg L^{-1}) which were transformed using log_{10} for improved visualisation. P < 0.001 means the correlation was highly significant

4.3.2 Variation in the *Sdh* genes of baseline isolates

DNA sequences of the *SdhB* (n = 93), *SdhC* (n = 89) and *SdhD* (n = 92) subunits in the baseline collection were compared. Only two nucleotide substitutions were observed in the *SdhB* subunit and neither altered the target protein. There was more variation in the *SdhC* subunit; 60 nucleotide substitutions were observed in the protein coding region, 49 of which were synonymous mutations, i.e. having no effect on the target protein, and 11 were non-synonymous, i.e. altering the target protein. While 36% of those isolates had no non-synonymous mutations, comparable to the reference wild-type IPO323, the remaining 64% of isolates had changes in the protein (Table 4.5a). In the *SdhD* subunit; 52 mutations were observed, 12 of which were non-synonymous (Table 4.5b), leading to protein changes in 10% of isolates. Three isolates had amino-acid changes in both *SdhC* and *SdhD* subunits. With all fungicides, including boscalid, the distribution of EC_{50} in the non-wild-type variants was similar to that of the wild-type EC_{50} values (Table 4.5).

Table 4.5 Summary of sensitivity profiles of (a) *SdhC* (n = 89) and (b) *SdhD* (n = 92) baseline *Zympseptoria tritici* variants

a

			Mean fungicide sensitivity[a] $log_{10}EC_{50}$ (mg L^{-1})					
SdhC variant		*n*	Bixafen	Boscalid	Fluopyram	Fluxapyroxad	Isopyrazam	Penthiopyrad
	Isolate 4465		-0.873	0.091	-0.135	-0.735	-0.600	-0.431
1	Wild-Type	32	-0.785	-0.209	-0.001	-0.842	-0.889	-0.836
			(-1.377 to -0.457)	(-1.180 to 0.180)	(-0.842 to 0.735)	(-1.602 to -0.407)	(-1.770 to -0.420)	(-1.481 to -0.427)
2	N33T*, N34T*	42	-0.622	-0.141	-0.003	-0.697	-0.699	-0.611
			(-1.237 to -0.085)	(-0.796 to 0.229)	(-0.996 to 0.804)	(-1.658 to -0.229)	(-1.523 to -0.146)	(-1.328 to 0.141)
3	N33T, N34T, R40C	1	-1.022	-0.654	-0.721	-1.161	-1.469	-1.194
4	N33T, N34T, V150L	1	-0.457	0.039	0.439	-0.355	-0.458	-0.551
5	I29V*	6	-0.652	0.051	0.059	-0.701	-0.419	-0.310
			(-1.387 to -0.184)	(-0.932 to 0.463)	(-0.590 to 0.3)	(-1.292 to -0.389)	(-1.509 to 0.154)	(-1.377 to 0.176)
6	F23S, I29V	1	-0.827	-0.029	-0.818	-0.886	-1.328	-1.155
7	R12W	2	-0.623	0.004	0.155	-0.532	-0.264	-0.400
			(-0.721 to -0.545)	(-0.073 to 0.067)	(0.042 to 0.242)	(-0.706 to -0.408)	(-1.416 to -0.153)	(-0.580 to -0.273)
8	A106V	1	-0.532	-0.020	-0.334	-0.703	-0.770	-0.662
9	P127A	1	-0.640	-0.298	-0.175	-0.569	-0.378	-0.179
10	K60R	1	-1.244	0.142	-0.633	-1.292	-1.310	-1.180
11	Q42P	1	-0.971	-0.135	0.453	-0.660	-0.943	-0.833

n number of times the *Sdh* variant was observed

[a] The mean of isolates where that *Sdh* variant was seen more than once, and underneath in parenthesis the range of those EC_{50} values. Otherwise, the EC_{50} of the single isolate with that *Sdh* variant is shown

*Individual mutations identified previously in the literature originating from field *Zymoseptoria tritici* isolates

Table 4.5 cont.

b

	SdhD variant	*n*	Mean fungicide sensitivity[a] $log_{10}EC_{50}$ (mg L^{-1})					
			Bixafen	Boscalid	Fluopyram	Fluxapyroxad	Isopyrazam	Penthiopyrad
	Isolate 4465		-0.873	0.091	-0.135	-0.735	-0.600	-0.431
1	Wild-Type	83	-0.680	-0.127	0.028	-0.733	-0.714	-0.631
			(-1.387 to -0.085)	(-1.180 to 0.463)	(-0.996 to 0.804)	(-1.602 to -0.229)	(-1.770 to 0.155)	(-1.481 to 0.176)
2	A5T, L11F, T25V, L26I, T34S	1	-0.500	-0.156	-0.143	-0.759	-0.730	-0.943
3	T18N	1	-1.009	-0.421	-0.539	-1.086	-0.987	-1.076
4	K186R	1	-0.693	-0.346	-0.177	-0.924	-0.762	-0.742
5	R33C	1	-0.504	-0.346	-0.043	-0.860	-0.947	-0.785
6	Q38R	1	-0.870	-0.359	-0.697	-1.167	-1.187	-1.155
7	K183E	1	-0.244	-0.298	-0.633	-1.292	-1.301	-1.174
8	T181A	2	-0.402	-0.033	0.107	-0.618	-0.799	-0.567
			(-1.032 to -0.155)	(-0.346 to 0.147)	(-0.793 to 0.379)	(-1.509 to -0.347)	(-1.420 to -0.551)	(-1.301 to -0.309)
9	T19N	1	-0.801	0.180	-0.419	-0.565	-0.854	-0.682

n number of times the *Sdh* variant is observed

[a] The mean of isolates where that *Sdh* variant was seen more than once, and underneath in parenthesis the range of those EC_{50} values. Otherwise, the EC_{50} of the single isolate with that *Sdh* variant is shown

4.3.3 Selection experiments

The sensitivity of 2297 mono-pycnidial *Z. tritici* isolates collected both pre- and post-fungicide treatment was tested (Table 4.6). Due to contamination or poor growth of some isolates, only 2292 isolates were tested for sensitivity to epoxiconazole and 2283 isolates were tested for sensitivity to isopyrazam. The pre-treatment collection (Pre-T) ranged in sensitivity to epoxiconazole from a $\log_{10}EC_{50}$ (mg L^{-1}) of -1.475 to 0.815 (variation factor of 195) and to isopyrazam from a $\log_{10}EC_{50}$ (mg L^{-1}) of -2.737 to 0.063 (variation factor of 632). Epoxiconazole sensitivity did not differ between locations before treatment (P = 0.3), but isopyrazam sensitivity did (P = 0.003); the pre-treatment sample from Drogheda was the most sensitive to isoyprazam and Julienstown, Killeagh and Oak Park 1 were the least sensitive (Figure 4.2). In treatments containing epoxiconazole, i.e. solo epoxiconazole and the mixture, larger shifts in sensitivity to epoxiconazole were measured compared to in treatments without (P = 0.002) (contrast 1, Table 4.7a). Similarly, treatments including isopyrazam saw larger shifts in sensitivity to isopyrazam than those without (P = 0.026) (contrast 1, Table 4.7b). Smaller shifts in sensitivity to epoxiconazole were observed after the full rate mixture in comparison to the full rate solo epoxiconazole (P = 0.015) (contrast 2, Table 4.7a), but there was no difference in isopyrazam sensitivity between the mixture and solo isopyrazam (P = 0.8) (contrast 2, Table 4.7b).

Table 4.6 Mean sensitivity ($log_{10}EC_{50}$ mg L^{-1}) from individual treatments in the selection experiment, including pre-treatment, over all locations to (a) epoxiconazole and (b) isopyrazam, and broken down into treatment means per location

a				Mean EC_{50} (log_{10} mg L^{-1}) per location (number of isolates per location)					
Treatment[a]	*n*	Mean EC_{50}	*SE*	Julienstown (*n* = 243)	Killeagh (*n* = 291)	Oak Park 1 (*n* = 274)	Drogheda (*n* = 481)	Midleton (*n* = 484)	Oak Park 2 (*n* = 519)
Pre-T	212	-0.464	0.081	-0.469	-0.422	-0.466	-0.356	-0.569	-0.434
Un-T	353	-0.219	0.078	-0.412	-0.405	-0.384	0.038	-0.011	-0.165
EE	357	0.079	0.078	0.088	-0.058	-0.172	0.265	0.052	0.332
ee	323	0.010	0.078	-0.106	-0.002	-0.269	0.076	0.218	0.146
II	339	-0.147	0.078	-0.253	-0.385	-0.346	0.105	0.162	-0.213
ii	176	-0.037	0.094	*	*	*	0.088	0.262	-0.093
EIEI	330	-0.099	0.078	-0.064	-0.233	-0.350	0.195	0.081	-0.170
eiei	202	-0.075	0.093	*	*	*	0.132	0.092	-0.108

Table 4.6 cont.

b				Mean EC_{50} (log_{10} mg L^{-1}) per location (number of isolates per location)					
Treatment[a]	*n*	Mean EC_{50}	*SE*	Julienstown (*n* = 243)	Killeagh (*n* = 283)	Oak Park 1 (*n* = 274)	Drogheda (*n* = 481)	Midleton (*n* = 483)	Oak Park 2 (*n* = 519)
Pre-T	212	-1.132	0.047	-1.078	-1.008	-1.033	-1.373	-1.201	-1.141
Un-T	353	-1.103	0.040	-1.184	-1.069	-1.080	-0.973	-1.188	-1.072
EE	358	-1.070	0.040	-1.155	-1.123	-1.052	-1.003	-0.950	-1.107
ee	323	-1.083	0.040	-1.134	-1.068	-1.153	-1.139	-0.879	-1.154
II	335	-1.011	0.040	-0.947	-0.891	-1.012	-0.993	-1.155	-1.118
ii	176	-1.042	0.056	*	*	*	-0.885	-1.095	-1.073
EIEI	325	-0.997	0.040	-1.026	-0.915	-1.071	-0.911	-0.959	-1.091
eiei	201	-0.993	0.055	*	*	*	-0.913	-1.054	-1.055

[a] Treatment information in Table 4.2. Briefly, Un-T= un-treated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; I or i: isopyrazam; uppercase: full dose; lowercase: half dose

n number of *Zymoseptoria tritici* samples per treatment and per location

*EC_{50} values not determined for these treatments at these location

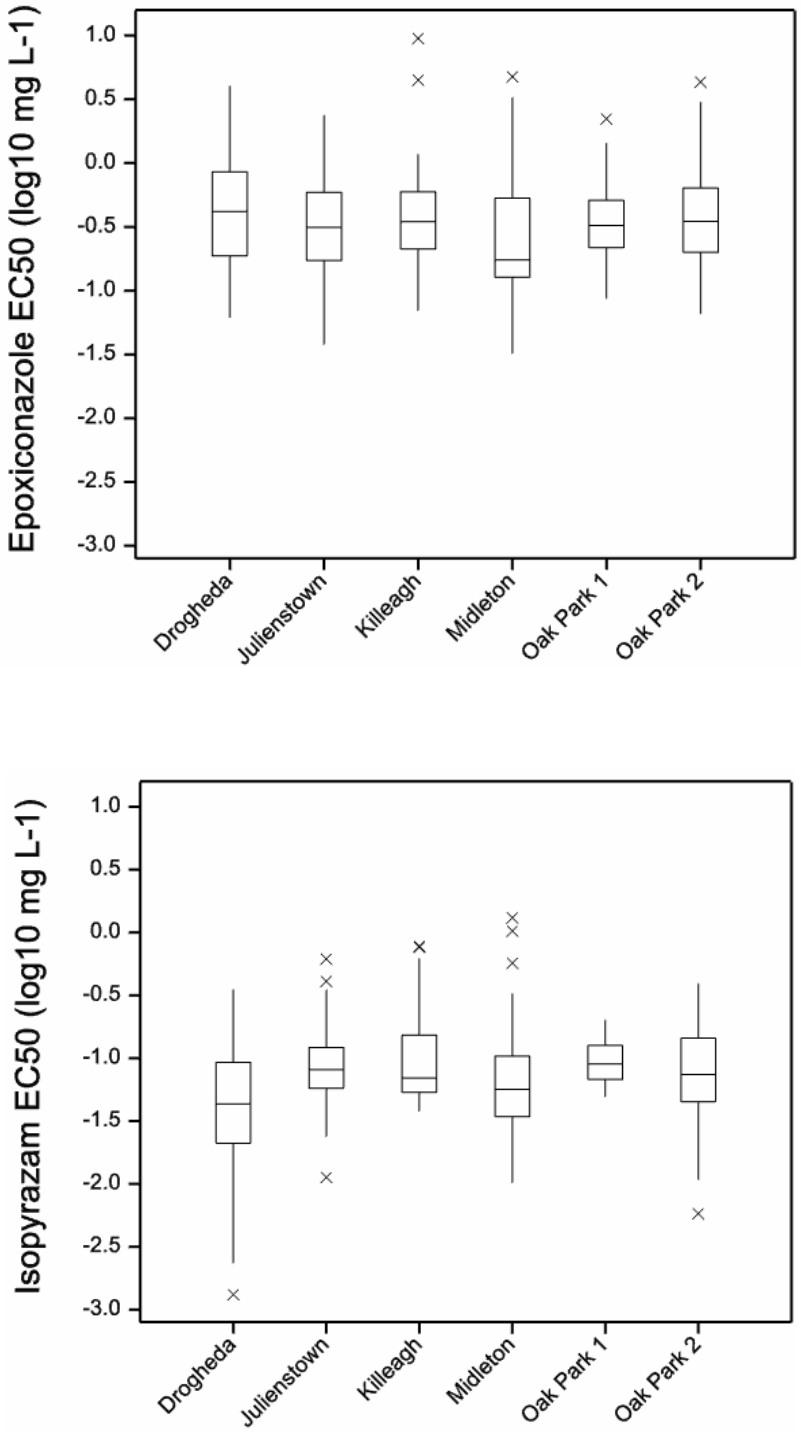


Figure 4.2 Distribution of $log_{10}EC_{50}$ values for epoxiconazole sensitivity (top) and isopyrazam sensitivity (bottom) from the pre-treatment collection sampled from each of the six locations, illustrated with box and whisker plots. The line through the box represents the median. Number of pre-treatment isolates tested from each site varied: Drogheda n = 39; Julienstown = 29, Killeagh = 20, Midleton = 44, Oak Park 1 = 21, Oak Park 2 = 59

Table 4.7 Independent single degree of freedom contrasts between treatments for (a) epoxiconazole and (b) isopyrazam sensitivity

a			Treatment coefficients* included in each contrast question						
Contrast	*Effect sizes*	*P*[a]	Un-T[b]	EE	ee	II	ii	EIEI	eiei
1. Treatments with any epoxiconazole cf. those without	0.086	0.002	-1	1	0	-1	0	1	0
2. Mixture cf. epoxiconazole solo	0.089	0.015	0	1	0	0	0	-1	0
3. Effect of dose	0.015	0.7	0	1	-1	1	-1	1	-1

b			Treatment coefficients* included in each contrast question						
Contrast	*Effect sizes*	*P*[a]	Un-T[b]	EE	ee	II	ii	EIEI	eiei
1. Treatments with any isopyrazam cf. those without	0.041	0.026	-1	-1	0	1	0	1	0
2. Mixture cf. isopyrazam solo	-0.007	0.8	0	0	0	1	0	-1	0
3. Effect of dose	-0.02	0.6	0	1	-1	1	-1	1	-1

a P-value is based on the F-distribution

b Treatment information in Table 4.2. Briefly, Un-T= un-treated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; I or i: isopyrazam; uppercase: full dose; lowercase: half dose

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

There were no significant effects of reducing dose on either epoxiconazole (P = 0.6) or isopyrazam (P = 0.7) sensitivity (contrast 3, Table 4.7a & Table 4.7b). Isopyrazam and epoxiconazole sensitivities were significantly (P < 0.001) and positively correlated, but that correlation was weak (r = 0.2) (Figure 4.3).

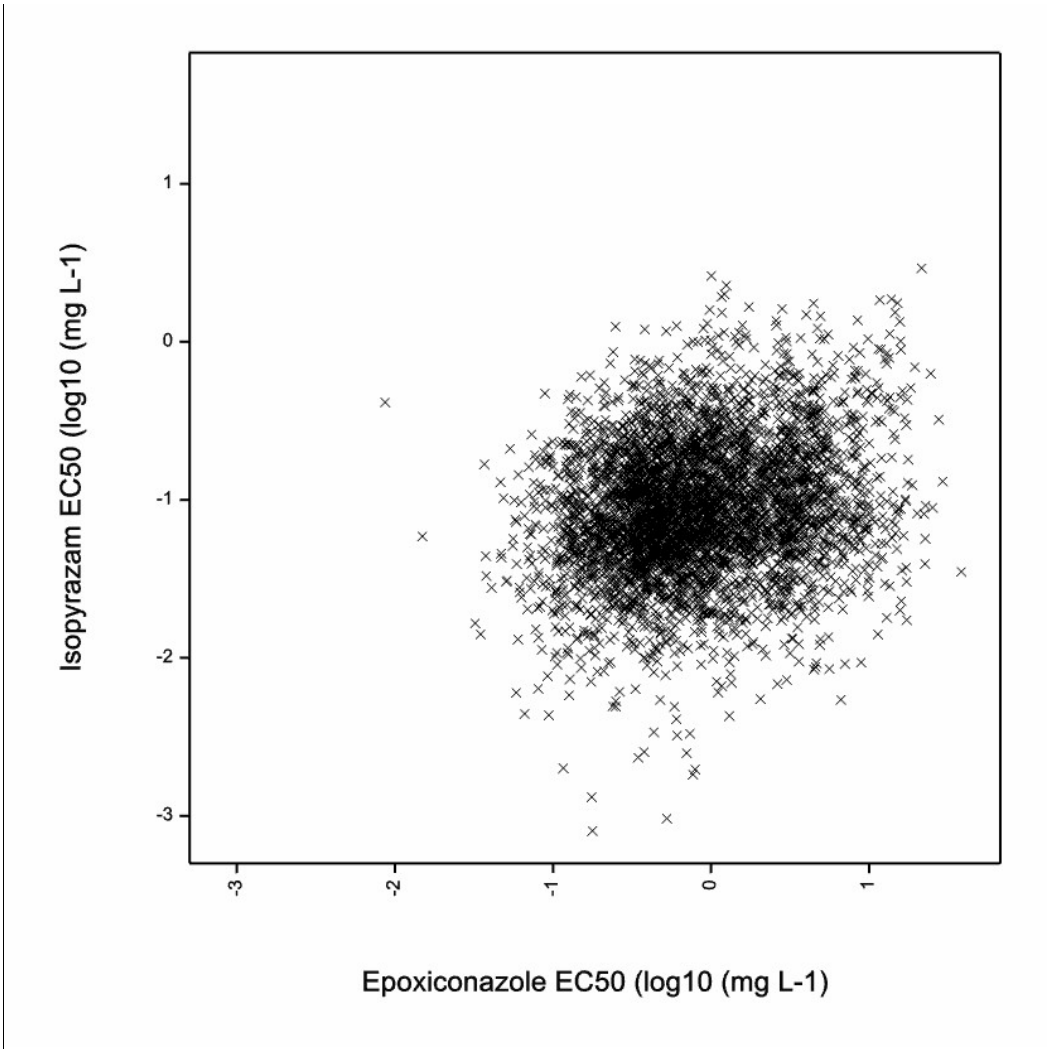


Figure 4.3 Scatter plot showing the correlation between isopyrazam and epoxiconazole sensitivity for isolates from the selection experiment: n = 2283, r = 0.2, *P* < 0.001

4.3.4 Disease severity and its relationship with selection

Un-treated control plots had significantly more disease than the treated with an overall mean of 11.6% (3.4 sqrt %) of the flag leaf area diseased at GS 71-83 ($P < 0.001$, Table 4.8). Levels of STB in the un-treated plots varied between locations: Midleton showed the highest severity with an average of 23% of leaf area infected (4.8 sqrt %) STB on flag leaves; lowest severity was at Oak Park 2 where flag leaves had an average of 1% of leaf area infected (1 sqrt %) STB. The performance of each product (full and half rate) depended greatly on location ($P < 0.001$, Table 4.8).

Table 4.8 Disease severity at GS 71-83 (measured as average percent STB per surface area of the flag leaf, square root transformed) between treatments at each location. Underneath are the results of a cross-location analysis using factorial plus control procedure

	Location						
Treatment [a]	Julienstown	Killeagh	Oak Park 1	Drogheda	Midleton	Oak Park 2	Mean
Un-T	4.74	4.63	3.07	2.13	4.81	1.01	3.4
EE	1.26	1.8	1.02	0.59	1.33	0.08	1.01
ee	2.67	1.9	1.24	1.01	1.46	0.2	1.41
II	0.53	0.67	0.9	0.12	2.58	0	0.8
ii	1.47	1.54	1.21	0.41	2.66	0	1.21
EIEI	0.92	1.08	1.25	0.15	0.72	0.03	0.69
eiei	1.57	1.94	1.5	0.16	1.6	0	1.13
Mean	1.88	1.94	1.45	0.65	2.17	0.19	1.38

Factorial plus control	*P*	LSD (5% level)
Control [b]	**< 0.001**	0.174
Location	**< 0.001**	0.427
Product[c]	**0.001**	0.198
Rate [d]	**< 0.001**	0.186
Location.Product	**< 0.001**	0.485
Location.Rate	**0.001**	0.459
Product.Rate	0.97	0.228
Location.Product.Rate	0.16	0.559

[a] Treatment information in Table 4.2. Briefly, Un-T= un-treated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; I or i: isopyrazam; uppercase: full dose; lowercase: half dose
[b] Control is all fungicides treatments compared to the un-treated control; EE+II+ee+ii+EIEI+eiei cf. Un-T
[c] Product is full and half rates of each treatment compared; EE+ee, II+ii, and EIEI+eiei
[d] Full rates cf. half rates; EE+II+EIEI cf. ee+ii+eiei

Epoxiconazole applied as a solo active ingredient gave the lowest level of control of all fungicides at all locations except at Midleton and Oak Park 1, where isopyrazam applied as a solo active ingredient and the mixture respectively provided the lowest levels of control (Table 4.8). The solo isopyrazam provided the best disease control at four of the locations, with the exception of Midleton and Drogheda, where the mixture performed best (Table 4.8). There was an effect of dose on disease ($P < 0.001$), but this varied between location ($P = 0.001$). The full dose applications provided better disease control than their half dose counterparts at all locations, but the difference between the two was not significant at every location (Table 4.8). There was an inverse relationship between disease severity and EC_{50} to both epoxiconazole and isopyrazam, and most of the variation was accounted for with the inclusion of location differences (Figure 4.4A, $R^2 = 0.59$, $P < 0.001$ and Figure 4.4 B, $R^2 = 0.50$, $P = 0.002$ respectively, common slope but intercepts differing between locations).

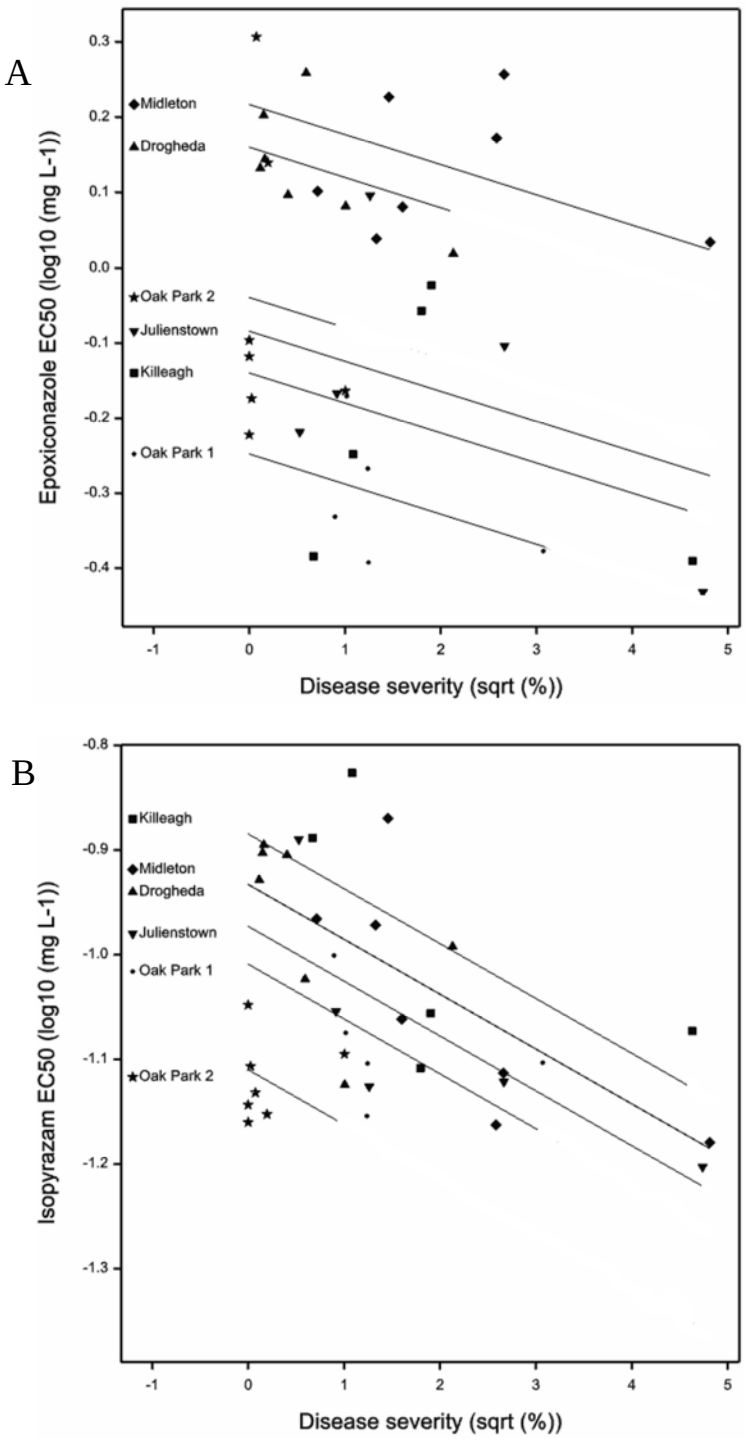


Figure 4.4 Fitted and observed relationship between disease severity (measured as average percent STB per flag leaf, square root transformed) and sensitivity (measured as $log_{10}EC_{50}$) (A) epoxiconazole, R^2 = 0.53, *P* < 0.001; common slope = -0.04; intercept for Killeagh = -0.14; Midleton = 0.217; Drogheda = 0.16; Julienstown = -0.084; Oak Park 1 = -0.248; Oak Park 2 = -0.039 and (B) isopyrazam sensitivity and disease severity R^2 = 0.44, *P* = 0.002, common slope = -0.052; intercept for Killeagh = -0.885; Midleton = -0.933; Drogheda = -0.931; Julienstown = -0.973; Oak Park 1 = -1.009; Oak Park 2 = -1.11

4.3.5 Effects of fungicides on yield

The un-treated control plots were lower yielding (7.05 t/ha) than the mean of all the fungicide treatments ($P < 0.001$, Table 4.9). Overall yield was significantly different between locations ($P < 0.001$, Table 4.9) with the 2013 locations (Oak Park 2, Midleton and Drogheda) yielding more than the 2012 locations (Oak Park 1, Julienstown and Killeagh). Yield improvements after fungicide application varied between locations ($P < 0.001$, Table 4.9). Oak Park 1 and Julienstown had improvements in yield of 2.5 and 1.9 t/ha respectively due to fungicides, and Oak Park 2 saw an improvement of just 0.2 t/ha. The product used had an effect on yield ($P = 0.028$, Table 4.9) with the solo isopyrazam and the mixture providing higher yields than the solo epoxiconazole. There was no significant difference in yield between full and half rates ($P = 0.2$, Table 4.9). There was a significant inverse relationship between disease and yield; but both the slope and intercept of this varied between locations (Figure 4.5, $R^2 = 0.96$, $P = 0.01$).

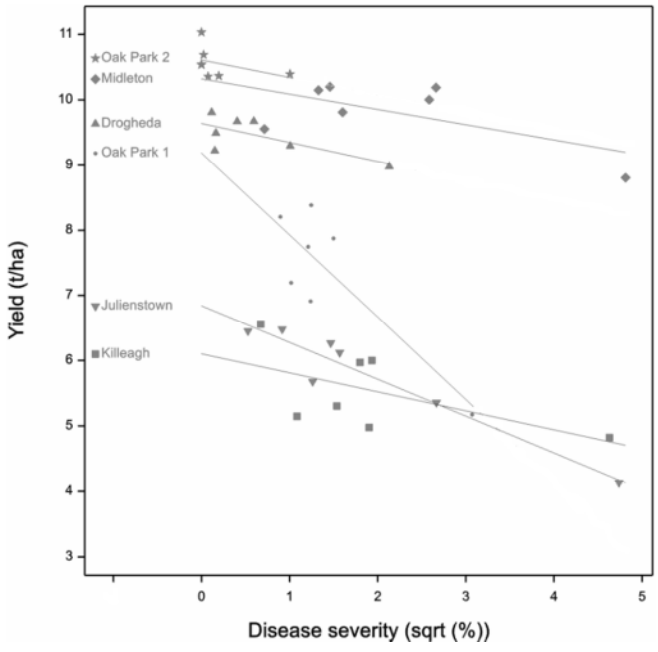


Figure 4.5 Fitted and observed relationship between disease severity (measured as average percent STB per flag leaf, (square root transformed) and yield (measured as tonne per hectare), R^2 =0.96, P = 0.013. Drogheda: y = 9.64 + -0.271x; Julienstown: y = 6.84 + -0.562x; Killeagh: y = 6.1 + -0.289x; Midleton: y = 10.32 + -0.233x; Oak Park 1: y = 9.2 + -1.261x; Oak Park 2: y =10.6 + -0.266x

Table 4.9 Yield (measured as tonne per hectare) between treatments at each location. Underneath are the results of a cross-location analysis using factorial plus control procedure

	Location						
Treatment [a]	Julienstown	Killeagh	Oak Park 1	Drogheda	Midleton	Oak Park 2	Mean
Un-T	4.13	4.82	5.17	8.98	8.81	10.39	7.05
EE	5.68	5.97	7.19	9.68	10.15	10.36	8.17
ee	5.36	4.98	6.91	9.3	10.2	10.36	7.85
II	6.45	6.55	8.21	9.81	10	10.54	8.59
ii	6.26	5.3	7.75	9.67	10.19	10.53	8.28
EIEI	6.48	5.15	8.38	9.22	9.56	10.69	8.25
eiei	6.12	6	7.87	9.49	9.81	11.03	8.39
Mean	5.78	5.54	7.35	9.45	9.82	10.56	8.08

Factorial plus control	*P*	LSD (5% level)
Control [b]	< **0.001**	0.349
Location	< **0.001**	0.855
Product [c]	**0.028**	0.396
Rate [d]	0.227	0.373
Location.Product	0.274	0.97
Location.Rate	0.64	0.914
Product.Rate	0.276	0.457
Location.Product.Rate	0.748	1.12

[a] Treatment information in Table 4.2. Briefly, Un-T= un-treated control, abbreviations denote the first and second sprays; E or e: epoxiconazole; I or i: isopyrazam; uppercase: full dose; lowercase: half dose
[b] Control is all fungicides treatments compared to the un-treated control; EE+II+ee+ii+EIEI+eiei cf. Un-T
[c] Product is full and half rates of each treatment compared; EE+ee, II+ii, and EIEI+eiei
[d] Full rates cf. half rates; EE+II+EIEI cf. ee+ii+eiei

4.3.6 Variation in the *Sdh* genes of isolates from the selection experiment

DNA sequences of the *SdhB* (n = 86), *SdhC* (n = 81) and *SdhD* (n = 96) subunits were determined in the experimental isolates least sensitive to isopyrazam. A single synonymous mutation was found in the *SdhB* subunit so all the experimental isolates had a wild-type *SdhB* subunit. In the *SdhC* subunit, 50 nucleotide mutations were found, seven of which were non-synonymous, and found in 68% of isolates (Table 4.10a). In the *SdhD* subunit; a total of 35 nucleotide mutations were found, two of which were non-synonymous and found in 2% of isolates (Table 4.10b). One isolate had amino-acid mutations in both the *SdhC* and *SdhD*

subunits. The EC_{50} distribution of isolates with *Sdh* variant sequences was similar to the EC_{50} distribution observed in the isolates with wild-type sequences, for both isopyrazam and epoxiconazole. Apart from the wild-type isolates, only a single *Sdh* variant was found in more than one isolate: N33T/N34T in the *SdhC* subunit was found in 49% of isolates but as with the other variants the EC_{50} values were spread over the entire range of both isopyrazam and epoxiconazole.

Table 4.10 Summary of sensitivity profiles of (a) *SdhC* (n = 81) and (b) *SdhD* (n = 89) variants from *Zymospetoria tritici* isolates from the selection experiment

a

			Mean fungicide sensitivity[a] $log_{10}EC_{50}$ (mg L^{-1})	
SdhC variant		*n*	Isopyrazam	Epoxiconazole
1	Wild-Type	34	-0.121	0.544
			(-0.384 to 0.359)	(-0.606 to 1.245)
2	N33T*, N34T*	40	-0.137	0.521
			(-0.376 to 0.368)	(-0.644 to 1.238)
3	L11I	1	-0.198	-0.040
4	I29V*,N33T, N34T	1	0.052	-0.166
5	N33T, N34T, V150L	1	-0.258	0.815
6	P127Q	1	-0.351	0.448
7	V48A	1	-0.302	0.482
8	I29V	2	-0.134	0.560
			(-0.344 to 0.007)	(-0.198 to 0.821)

b

			Mean fungicide sensitivity[a] $log_{10}EC_{50}$ (mg L^{-1})	
SdhD variant		*n*	Isopyrazam	Epoxiconazole
1	Wild-Type	94	-0.130	0.580
			(-1.377 to 0.368)	(-0.664 to 1.245)
2	K186R	1	-0.108	0.339
3	L32I	1	-0.345	-0.396

[a] The mean of isolates where that *Sdh* variant was seen more than once, and underneath in parenthesis the range of those EC_{50} values. Otherwise, the EC_{50} of the single isolate with that *Sdh* variant is shown
n number of times the *Sdh* variant is observed
*Individual mutations identified previously in the literature (Fraaije *et al*., 2012) originating from field *Zymoseptoria tritici* isolates

4.4 Discussion

Between the 207 baseline isolates and approximately 2300 experimental isolates of *Z. tritici* sampled over two recent seasons, there is no evidence to suggest that any level of resistance to SDHIs has yet developed in Irish field populations. The different rates of intrinsic sensitivity observed to the different SDHI active ingredients do not necessarily suggest that some products are more or less effective than others, but were likely seen because of differences in the size and structure of each molecule and how they subsequently interact with the pathogen (Scalliet *et al*., 2011). A broad range of sensitivities amongst the baseline *Z. tritici* collection was demonstrated for all SDHI fungicides tested in this study. The variation factors were higher in the current collection for bixafen, boscalid and isopyrazam, but means were similar, to those in comparable work from Fraaije *et al*. (2012) and Schürch and Cordette (2013). Like the baseline collection, the experimental locations had a high level of EC_{50} variation prior to the application of fungicide treatment. Location differences were few, but where present they serve to highlight the natural variation in the population as a whole.

The relationships between the different SDHIs as observed in the *Z. tritici* baseline studies are in agreement with Fraaije *et al*. (2012) who found clear positive correlations between bixafen and isopyrazam, boscalid and isopyrazam and bixafen and boscalid, and those of Schürch and Cordette (2013) who found similar patterns but with weaker relationships. The relatively weak correlation between fluopyram and the other SDHIs observed have also been reported in other plant pathogens, although mostly in SDHI resistant isolates (Avenot & Michailides, 2010, Ishii *et al.*, 2011). Gudmestad *et al.* (2013) also observed similar disparities in correlations between fluopyram and boscalid and penthiopyrad in baseline collections of *Alternaria solani* and suggested that fluopyram might bind at a different site on the iron-sulphur protein, or somewhere else in complex II. However, Fraiije *et al.* (2012) and Scalliet *et al.* (2012) both confirmed through molecular docking modelling

that fluopyram binds to the same site in the succinate dehydrogenase as the other SDHIs. Fraiije *et al.* (2012) suggest that due to the addition of a benzamide moiety in fluopyram it is likely to have extended binding in the quinone binding site giving it a lower binding energy than the other SDHIs (Fraaije *et al*., 2012). Amongst the SDHI resistant lab mutants generated by Scalliet *et al.* (2012) those resistant to fluopyram contained target site substitutions not found in the mutants resistant to the other SDHIs. The authors suggest that this may be due to the specific structure of fluopyram and how it interacts with the *Sdh* complex. The absence of substitutions in the *SdhB, C* or *D* subunits believed to affect sensitivity to any of the SDHIs in the baseline isolates in this study suggests that the lack of cross-resistance between fluopyram and the other SDHIs is not due to differences in the binding of the fungicides to the succinate dehydrogenase. Instead, the differences observed may reflect differences in the capacities in *Z. tritici* to extrude fluopyram and the other SDHIs from its cells. Zwiers *et al*. (2002) and Roohparvar *et al.* (2007) have highlighted the ability of *Z. tritici* to protect itself against fungicides (DMIs and QoIs) by secreting them using membrane bound transporters. As differences exist in the molecular structures of the SDHI, and in particular fluopyram, such differential secretion of fungicides from the same group is feasible. Even-though strong cross-resistances exist between the other SDHIs, both Fraaije *et al*. (2012) and Scalliet *et al*. (2012) also found that different SDHIs selected specific mutations in the binding pocket of *Z. tritici*. Therefore, once such mutations arise in a *Z. tritici* population the strong cross-resistance patterns present in the baseline isolates may change.

The lack of amino-acid variation in the *SdhB* subunit is contrary to most other SDHI resistance studies of field or mutated strains of *Z. tritici*, where the *SdhB* subunit has been variable. Further, mutations in the *SdhB* subunit have been shown to contribute considerably to resistance development (Skinner *et al*., 1998, Fraaije *et al*., 2012, Scalliet *et al*., 2012).

Even so, in the current study, as in Fraaije *et al*. (2012), the *SdhC* subunit was the most diverse in the gene. No isolates in either the baseline collection or the experimental field collection had any known resistance-conferring amino-acid changes, and few out-of-WT-range EC_{50} values were found in either baseline or experimental isolates. Those mutations which were present appeared to be neutral; EC_{50} values were within the wild-type range and, while having no apparent effect on the fitness of the isolate in the presence of a fungicide, the mutations still persist in the population. In addition, mutations were not found in or close to the putative *Sdh* binding pocket as described by Fraaije *et al*. (2012) and Scalliet *et al*. (2012), indicating that they have no direct effect on binding/fungicidal activity. This is despite the fact that the baseline isolates may have been exposed to boscalid (as it has been commercially used in Ireland since 2005) and the isolates within the selection experiment collection were chosen because they had the highest levels of isopyrazam EC_{50} values available from the whole collection. The absence of mutations in field isolates which were found in mutagenesis studies (Skinner *et al*., 1998, Fraaije *et al*., 2012, Scalliet *et al*., 2012) suggests that either the mutations have not actually occurred, or that the changes brought about in *Z. tritici* by mutagenesis may have no overall selective advantage in the field. Reduced enzyme activity in some of the mutated strains, as demonstrated by Scalliet *et al*. (2012), may contribute to reduced fitness.

The weak cross-resistance and the differential selection observed between isopyrazam and epoxiconazole was expected as each a.i. targets a different site. This suggests that strains resistant to one a.i. are only weakly selected by the other and demonstrates that these two a.i.s are currently suitable for mixing. Much work has been done on the effects of mixtures on selection for resistance. Hobbelen *et al*. (2013) provide a short review of the literature. Many studies include a combination of two at-risk fungicides, but only report the effects on one; whichever one the authors class as the most at risk of resistance. Ensuring that SDHIs are

mixed with another mode-of-action, such as an azole, is technically for the benefit of the SDHI, but the addition of the SDHI component to the azole undoubtedly provides a level of resistance protection for the azole component. In the isopyrazam/epoxiconazole mixture described in this study, and indeed any other SDHI/azole mixture, it can be difficult to assess which component is the most at-risk fungicide or which is the most important to protect. In the context of resistance management, both a.i.s can be classed as at-risk; isopyrazam because of the high potential for development of *Z. tritici* resistance (Fraaije *et al*., 2012) and epoxiconazole because insensitivity is already present in populations (Stammler & Semar, 2011). If we assumed that isopyrazam was the more at-risk of the two components, the current results would appear to contradict Hobbelen *et al*. (2013) who hypothesise that the more at-risk fungicide would be protected in a combination of two high-risk fungicides. We found that the addition of epoxiconazole had no effect on isopyrazam sensitivity, with all treatments containing isopyrazam showing reduced sensitivity to the SDHI compared to those without. However, as the treated collections were still very sensitive to isopyrazam, to suggest that adding epoxiconazole to isopyrazam would never be of any benefit to the isopyrazam component would be misleading, and whether the same results would have been observed in a less sensitive population remains to be determined.

Based on the spectrum of activity and the track record of longevity of the azoles, the azole component is as important as the SDHI component and should be protected. The frequency of strains with reduced sensitivity to epoxiconazole, which has increased over the past decade because of the emergence and selection for less sensitive *CYP51* variants (Stammler & Semar, 2011), suggests that this portion of the population is in the adjustment phase of resistance evolution where, depending on the situation, the recommended amounts of a.i. may not control STB to an acceptable level. We saw this in the field experiments where plots treated with epoxiconazole showed the highest frequency of epoxiconazole insensitive

Z. tritici, in addition to being the least effective of the fungicides for STB control and providing the lowest eventual yield of all the fungicides tested. Currently the azole insensitive isolates remain sensitive to the SDHIs, and even though some selection for reduced epoxiconazole sensitivity did occur after the mixture with isopyrazam, the addition of isopyrazam to epoxiconazole had a large buffering effect on the level of that selection.

One of the caveats when mixing two fungicides as an anti-resistance strategy is that the individual components of a mixture should be included at a rate which provides effective control when applied alone (Anon, 2013b). If the proportion of epoxiconazole in the mixture could be increased to a level where it provided good control, it may provide more protection for the SDHI, however in the present study epoxiconazole was applied in the mixture at the recommended label rate and any further increases may adversely increase selection for azole resistance. Alternatively, mixing the SDHI with a more effective azole might improve the situation. Combining the only non-cross-resistant SDHI, fluopyram, with isopyrazam may be an option to protect the SDHI and azole component, but this is a short term solution which is likely to speed up the selection for resistance once it emerges, and so not to be recommended. If this option were to be utilised, alternating the SDHIs instead of mixing might be the better option. Alternating two products which contain different SDHIs limits the exposure of the population to the same SDHI to once per season, which reduces the selection coefficient (van den Bosch *et al*., 2014). The ideal option for protecting both the SDHI and azole components would be however, to add a fungicide which is at a low risk-of-resistance, i.e. a multi-site, such as chlorothalonil or folpet. The multi-sites are protectants which work best to prevent disease, and are most effective at the earliest stages of disease development. Early in the season, in a low disease pressure situation, a multi-site applied as a solo product can be effective. In high disease pressure and in curative situations, the multi-sites, if used alone, may not be the best option (compared to azoles and SDHIs) (Anon, 2013a), but used in

mixtures with azoles or SDHIs and at robust rates (Anon, 2014b), they prevent disease, protect the partner fungicide from resistance (Kildea & Glynn, 2014) and have themselves yet to be affected by resistance. Hobbelen *et al*. (2014) hypothesised that mixing a high-risk and a low-risk fungicide would increase the time to emergence of resistance to the high-risk fungicide.

Aiming for perfect control is not always necessary in order to maintain yields. In comparison to the full dose treatments, reduced doses significantly reduced the amount of disease control, but had no effect on yield, or indeed selection for isolates with reduced sensitivity. This goes against most studies on this subject, where reducing the dose usually reduces selection (Van den Bosch *et al*., 2011). In Chapter 2, we saw similar results when azoles were applied as solo treatments or in combination with other azoles, and concluded that azole insensitivity was possibly in the adjustment phase of resistance development and the lower doses were not enough to control STB when used alone. The effect of dose on the emergence of resistance is less studied. Van den Bosch *et al*., (2014) hypothesise that a higher dose may alter the emergence phase of evolution: the rate of the appearance of mutations is relative to the size of the population and in theory, if the population is kept to low numbers by using higher doses, fewer mutations should arise.

This study provides evidence for no SDHI resistance in the current population, but as long as SDHIs are in use it should be assumed that mutations conferring insensitivity will arise in the future and continuous monitoring of populations is necessary to avoid field control failures. Anti-resistance strategies are also necessary to avoid field control failures. In this instance we found that mixing an SDHI with a different mode-of-action did not seem to have the desired effect. However, it is possible that result arose due to the highly sensitive population, which could have led to no apparent benefit of adding azoles. However, the benefit of adding the SDHI to the azole was clear. In order to protect both groups of

fungicides from resistance, it would be prudent to limit the number of applications of individual a.i.s from each group to once per season. However, if it is necessary to use multiple applications from a single fungicide group in one season, alternation between a.i. within a group should reduce selection pressure. Moreover, the addition of a multi-site fungicide would add protection for the SDHI while not affecting selection for resistance in any way.

Chapter 5: General discussion

This PhD project arose because of the continuing decrease in azole sensitivity, and the prospect of managing cereal crops without them. The aim of this work was to investigate the effects of combining different fungicides on fungicide sensitivity, and on changes in the fungicide target protein. A reduction in the frequency of isolates with reduced azole sensitivity was brought about by reducing the number of applications of a specific active ingredient through i) alternating two azoles, ii) mixing an azole with an SDHI. Mixing two azoles did not reduce the frequency of isolates with reduced azole sensitivity.

The three experimental chapters in this thesis cover three separate but connected subjects. Chapter 2 investigated the effects of combining two azoles, and of reducing recommended dose rates of these combinations, on azole sensitivity. This was a large experiment, and provides a solid contribution to this field of research. While much experimental work has been done on combining fungicides, few of those studies look at the effects of alternating fungicides (sequential application of different fungicides) and even fewer study the effects of mixing azoles - this work tackles both. Chapter 3 was a direct extension of Chapter 2, and studied the exact target protein changes brought about by applying solo azoles and a mixture of azoles. This is the first large scale study of the complete *CYP51* gene of Irish *Z. tritici* populations and will contribute to the growing body of work on the evolution of azole resistance. Finally, Chapter 4 moved on to study the effects that combining fungicides with different modes-of-action, an azole and a SDHI, would have on azole and SDHI sensitivity and on *Sdh* target protein changes. It combines three separate studies on fungicide sensitivity; i) the establishment of baseline sensitivity levels to new SDHI fungicides; ii) the determination of the effects of mixing SDHIs and azoles on fungicide sensitivity; and iii) the determination of the genetic diversity in the *Sdh* target gene in baseline and experimental isolates.

Fungicide resistance management is about getting a balance between disease control and selection for resistance, and can entail a trade-off; specifically, lower fungicide use can lead to a reduction in selection pressure, but may also mean a reduction in disease control. However this reduction in disease control should not always be viewed as leading to a reduction in yield. In Chapter 2, the azole mixture (epoxiconazole and metconazole) controlled STB significantly better than either the alternations or solo products, but there was no statistical difference between yields. This demonstrates that aiming for perfect control is not necessary, and additionally, in some cases may be uneconomical. A good level of control is however necessary. In this study when the recommended dose rates of the azole based fungicide combinations were halved and compared to the full doses, smaller shifts in sensitivity were observed, but were not significant, while disease control and yield were reduced significantly. So, the half rates of those fungicides were of limited practical use. However, extrapolation of that result to other fungicides in other pathosystems is not necessarily appropriate. Azole insensitivity in *Z. tritici* has evolved to the extent that management is now in the adjustment phase, where full recommended doses, and possibly increased doses, are needed for effective control. The work here was limited to the study of two doses, half and full recommended dose rates. Had the resources been available, another dose rate, for example ¾ of the full recommended amount, may have shown better disease control results. SDHI resistance on the other hand is in the pre-emergence phase of resistance where reduced application rates can provide effective control so as not to affect yield (as seen in Chapter 4) as well as possibly slowing the emergence of resistance (Shaw, 2009). Hobbelen *et al*. (2014) discuss two opposing effects of dose on the emergence of resistant strains: high doses may keep the population small which will reduce the number of mutations per unit time, but they will also reduce the competition between the sensitive and resistant strains, which will increase the chances of the resistant strain emerging. They conclude that

within the range of doses used in a commercial situation, dose rate had no effect on the emergence time of resistance. Nevertheless, if resistant strains were to emerge, reduced rates would be expected to lessen the selection pressure compared to full rates (Van den Bosch *et al*., 2011).

A recent review by van den Bosch *et al*. (2014) highlighted that most of the evidence on the subject concluded that mixtures can slow the selection for resistant strains. The two components of the azole mixture used in these experiments have in the past been shown to select differentially (Fraaije *et al*., 2007, Leroux *et al*., 2007, Kildea, 2009), and even though results in Chapter 2 confirm that is still the case, high levels of cross-resistance between both azoles existed in these *Z. tritici* populations. It was established that this mixture of epoxiconazole and metconazole did not have the effect of reducing selection for isolates with reduced sensitivity, but instead increased selection for isolates with reduced sensitivity to both fungicides. Correspondingly, from the *CYP51* alterations and variants which were positively selected by epoxiconazole or metconazole, most were positively selected by the mixture. This mixture is still commonly used, and it might be expected that the dual selection caused by mixing epoxiconazole and metconazole will lead to a faster evolution towards resistance to both components. This however does not spell the end for this or other azole mixtures. Firstly, azole mixtures still provide effective disease control, although how long this may last is questionable given the presence of strains in the Irish *Z. tritici* population with high levels of insensitivity to both fungicides. Secondly, it is possible that if we studied a different combination of azole fungicides the results may be different, but given the high evolutionary potential of *Z tritici* populations, any advantages of mixing azoles are likely to be temporary. Finally, the addition of an a.i. with a different mode-of-action to azole mixtures would protect the azole components at the same time as adding disease control, and is recommended by both FRAC and FRAG-UK.

Results from Chapter 2 demonstrated that if two azoles are to be combined in an effort to reduce selection for insensitive *Z. tritici* isolates while not adversely affecting yield, alternating, and in doing so limiting the application of either fungicide to once per season, would be the more successful tactic; however, the success of alternating two azoles was only applicable to the a.i. applied first in the alternation. Restricting the application of individual azoles to only once per season demonstrates the importance of maintaining and utilising the full range of azoles currently available. Further, the example of prochloraz could be used to initiate studies which might lead to the improved formulation of some older azoles or to the use of older azoles in new combinations - providing a new outlook for those fungicides and STB control. However, as most recent studies have shown, the rate at which the *Z. tritici* populations are evolving towards insensitivity highlights that the continued mixing of azoles is a short term solution which will accelerate selection for resistance. The method of sampling the experimental plots as previously discussed may have affected this result, i.e. the leaf layer sampled was treated with only the T2 fungicide and it could be maintained that the isolates collected were only affected by that one fungicide. However, it might be counter argued that as the disease is known to spread vertically through the crop canopy (Baccar *et al*., 2011), aside from ascospores coming in from outside the crop, most inoculum found on the flag leaf, and therefore directly affected by the T2 fungicide, would have been pre-selected by the T1 fungicide.

Considering that the same effect of alternating would probably apply to the sequential use of any two differentially selecting azoles, it would be a sensible approach to apply the more at-risk azole only once per season, and at the T1 application timing. Of the four standard fungicide timings T0, T1, T2 and T3, a consistently high yield response to the T2 (Anon, 2014c) means that that application provides the best return on investment, and so the more disease you can control at that time, the bigger the return. Hence, the most effective

product should be used at T2. In this instance, where epoxiconazole and metconazole were as effective as each other, it made little difference to disease control or yield which azole was applied at T1 or T2. However, when applying fungicides with different levels of efficacy, and assuming there are resistant strains present in the population, the more effective fungicide when applied at T2, is also likely to provide the strongest selection pressure compared to applying the more effective fungicide at T1. This takes us back to the importance of getting the correct balance between disease control and selection.

Even though limiting the number of applications of a specific azole can help slow the selection for strains with reduced sensitivity, as long as azoles are being used, *Z. tritici* populations will probably continue to evolve towards resistance. But do *in-vitro* resistance studies really reflect what is actually happening in the field? Some workers (Stammler *et al*., 2008, Strobel *et al*., 2014) suggested that field efficacy of epoxiconazole has seldom been compromised in the presence of amino-acid alterations that reduce the *in-vitro* sensitivity. However, Kildea (2009) showed that in 2006-07 epoxiconazole was significantly more effective than metconazole. In comparison to the current work, where the two fungicides were very similar, this indicates a loss of efficacy in epoxiconazole since then. Indeed, collaborative studies carried out in the UK by the HGCA and partners (Anon, 2014) saw a decrease in performance in azoles over the last decade. To slow the worsening of this situation and to maintain/extend the effective life of both epoxiconazole and metconazole, the addition of other modes-of-action to this and other azole mixtures is necessary. Indeed, azoles are currently seldom used without the protection of another mode-of-action.

With the decline in efficacy of the azoles in recent years, keeping on top of STB with the aim of avoiding a highly curative situation would help to maintain the effective life of azoles. However, as wet weather conditions in Ireland regularly inhibit the timely application of fungicides, they are often applied in situations which are in need of strong curative activity,

and so azoles which have curative activity are often necessary at T1 and T2. At these treatment times, when there are multiple generations of *Z. tritici* in the field, an additional mode-of-action which will protect the leaves, as well as provide a curative activity - to cover the loss of azole efficacy - is required. Mixing with an SDHI is currently one of the best options for disease control and protection of the azoles at these application timings. The addition of isopyrazam to epoxiconazole reduced the shift in epoxiconazole sensitivity compared to the solo products. However, the benefit was not seen both ways. It is possible that if SDHI resistant strains do emerge, the benefit of adding the azole may become more apparent. However, a couple points of caution should be noted here. In comparison to the Gleam treatments (the pre-formulated epoxiconazole + metconazole mixture), the Seguris treatments (the pre-formulated epoxiconazole + isopyrazam mixture) contained less epoxiconazole. Mixture components should be included at rates which are effective when used alone, and we saw in Chapter 2 that reduced rates of solo epoxiconazole were not effective when used alone. The lower amount of epoxiconazole in the azole/SDHI mixture (an amount of 3/4 the a.i. in the solo epoxiconazole) likely reduced the level of persistence of epoxiconazole. This would have reduced the exposure time, having the effect of reducing selection for strains with reduced epoxiconazole sensitivity. Conversely, it would have had the effect of increasing the time that populations were exposed to the isolated SDHI. In the instance of a high- plus low-risk mixture, reducing the dose of the high-risk or increasing the dose of the low-risk fungicide has been shown to reduce the selection pressure on the high-risk fungicide (Hobbelen *et al*., 2013). The components of the azole/SDHI mixture however, are two at-risk fungicides. Few studies on this type of mixture have been carried out (van den Bosch *et al*., 2014b), but in order to avoid unintentional exposure of one component to resistance, it would be safest to mix components which have similar levels of persistence (Shaw, 1993). Hence, one might speculate that if SDHI resistance strains were to arise, a

more effective rate of epoxiconazole in this mixture would be needed to control them. However, that is assuming that SDHI resistance will arise in otherwise sensitive strains. What happens if SDHI resistance arises in azole insensitive strains? Indeed, Fillinger *et al*. (2014) showed that it is possible, if not likely, that such field strains will emerge. Consideration should be given to how such strains would be controlled and what their impact on wheat production would be.

If SDHIs are mixed in the same way that azoles are mixed, how would that affect the development of resistance? In the baseline SDHI study, the level of cross-resistance between most of the SDHIs provided evidence that those SDHIs should not be mixed together. Conversely, the lack of cross-resistance between fluopyram and the other SDHIs might be a characteristic which in theory could be exploited. Either way, mixing SDHIs should not be viewed as an anti-resistance tactic, and should be done with caution (Anon, 2014a). The Fungicide Resistance Action Group (FRAG-UK) publishes guidelines each year in line with current research, and they suggest that tank mixtures of two SDHIs should be applied in a balanced mixture, and always with another fungicide with a different mode-of-action which provides equivalent disease control (Anon, 2014a). It is not made clear however, if the two SDHIs can or should be added at the full solo rates, or if reducing the rates to the equivalent of a single SDHI application is the best option. Shaw (1993) suggests that the latter option would be best, and the current chair of FRAG-UK confirmed that the latter approach is best (F. Burnett, personal communication). This should be clarified in the guidelines. Ultimately, with the knowledge gained from these azole experiments, combined with the potential for SDHI resistance to emerge (Fraaije *et al*., 2012) and for insensitive *Z. tritici* strains to re-combine (Brunner *et al*., 2008), SDHI mixtures need to be managed carefully from the outset, by limiting the total amount of SDHI active ingredient to the equivalent of a solo product and by adding a mixing partner with a different mode-of-action.

Between 2008 and 2010, there was evidence that shifts in azole sensitivity had stabilised (Stammler *et al*., 2008, Clark *et al*., 2010). However, the number of *CYP51* variants found in the most recent studies of European strains (Buitrago *et al*., 2014) and the collection in this study is an indication that the population has continued to evolve towards azole resistance. This continued evolution means that constant updating of the research and recommendations is a requirement for continuation of disease control and for resistance management guidelines. In the early days of resistance monitoring it was hoped that monitoring single amino-acid alterations could be used to make decisions regarding fungicide application programs or to predict field control. However, the high levels of variation in *Z. tritici* populations now makes that approach inadequate, and more complex molecular diagnostic solutions are needed for these complex populations. It was previously thought that managing wheat stubble, and so reducing the available primary inoculum, would help to reduce gene-flow of azole insensitive alleles on a regional level (Linde *et al*., 2002). However, parallel evolution of such alleles into different genetic backgrounds appears to be a normal occurrence, and circumvents attempts to reduce their spread through mechanical means. This highlights the importance of tackling the evolution of *Z. tritici* populations on a wide scale, nationally, and internationally where possible. There are regions with higher disease pressure and more diverse populations, and strains from those populations will eventually spread or re-emerge. Lessons can be learned from these high disease pressure areas.

For the moment, even though variation in the *Sdh* genes is present, it has no effect on sensitivity. But, how long until resistant alleles emerge? Once they do emerge, they may follow the same step-wise pattern as azole insensitive alleles, and the most recent survey data suggest that this is a possibility. However, earlier experimental evidence suggests otherwise: as with many other pathogens, *Z. tritici* populations may develop resistance to SDHIs which

is an all-consuming single-gene-no-fitness-cost resistance (Leroux & Walker, 2011), similar to QoI resistance, and protecting SDHIs from that scenario is very important for wheat production. Even though it has been speculated that the further accumulation of *CYP51* alterations might be restricted (Mullins *et al*., 2011), the evidence for recombination between *CYP51* variants, as well as the presence of alternative resistance mechanisms, strongly suggests that these populations are likely to continue evolving. Additionally, azoles are now not only threatened by resistance, but also by legislation. European Regulation 1107/2009 has set out to improve agricultural systems while protecting human health and the environment (Jess *et al*., 2014). Approval of products will move from a risk based to hazard based decision system, and in the best case scenario, 16 products which are currently approved for use in Ireland are likely to be withdrawn (Jess *et al*., 2014), which potentially include some azoles and the multi-site chlorothalonil. In the long term, if azoles are removed from the approved list, Europe-wide production of winter wheat and wheat self-sufficiency is likely to decrease significantly (Di Tullio *et al*., 2012). In the short term, the loss of azoles through either means would leave the SDHIs more exposed to resistance development.

Yield response to fungicide treatments in high disease pressure situations can be as high as 5 t/ha (Blake, 2011), suggesting that the continuation of winter wheat production in Ireland and other wheat growing regions of Europe is, in the immediate term at least, reliant on the availability of effective fungicides. If azoles were to become unavailable for control of STB, it would not be long before winter wheat production in Ireland would become unsustainable. Winter wheat crops could be replaced by winter/spring barley without much effort or capital. However, barley production is in itself at risk of losing the azoles which are used to control pathogens such as *Rhyncosporium commune*, *Ramularia collo-cygni* and *Pyrenophora teres*. Unlike *Z. tritici* though, the strobilurins, morpholines, and cyprodinyl are

all available and effective against the main disease of barley, *R. commune*, suggesting that the loss of azoles will not impact barley production as quickly as it will impact wheat production.

The loss of wheat production in Ireland is the worst case scenario. We have relied on fungicides for disease control for a long time, but other tools for managing STB are available. Collectively known as integrated pest management (IPM), with the potential loss of azoles we are being reminded of crop management practices which were once taken for granted, and which are now necessary for sustainable crop production. Indeed, European Parliament Directive 2009/128/EC requires each member country to draw up and act upon a plan for the sustainable use of pesticides (National Action Plan, NAP), which should include a plan for the promotion and adoption of IPM practices. Integrated pest management utilises all available crop protection resources and crop management techniques. It is undertaken with a view to reducing the disease pressure in the crop and our reliance on pesticides, and thus protecting the environment while reducing inputs and associated costs. The principal method of managing STB should be host variety choice. Taking into consideration local conditions and expected disease pressure, STB host resistance, while not currently perfect, can reduce the disease control burden on fungicides by reducing disease development, especially used in a high disease pressure situation. The benefits of using good host resistance would also be evident in situations where a grower has land some distance from the yard and in other situations where precise application timings are not likely to be achieved. The drawback to relying on host resistance is that varieties resistant to one disease may be susceptible to another or have undesirable physiological traits (Brown, 2002). The search for useful STB-resistant wheat germplasm is on-going. However, to be meaningful, research into and subsequent development of varieties for STB resistance requires a multifaceted approach (O'Driscoll *et al.*, 2014). Use of many partial-resistance genes: pyramiding two or more R genes, and mutating susceptibility genes, or even a combination of all three methods, would

provide a more solid base for STB host resistance (O'Driscoll *et al*., 2014). More generally, integrated management of STB might include the following strategies: management of crop canopy in order to reduce horizontal or vertical transfer of disease (Arraiano *et al*., 2009), achieved through manipulation of seeding rates, N management and variety choice based on physiological traits; reducing the green bridge in order to reduce early incidence of inoculum (Suffert *et al*., 2011), achieved through stubble management, manipulation of sowing date and host resistance; moving away from prophylactic spraying by making use of scientifically sound forecasting and early diagnosis systems (Burke & Dunne, 2008), as well as designing fungicide programmes which could be tailored around knowledge of the genetic makeup of local populations. Additionally, biological agents for the control of wheat leaf spot diseases are used successfully in some countries - such as Argentina (Perelló *et al*., 2009), and initial studies have been carried out on the potential of biological control agents for management of *Z. tritici* in an Irish setting (Kildea *et al*., 2008). However, poor efficacy of products outside of the controlled environment (Kildea *et al*., 2008) suggests that further studies are necessary before bio-control products will be accepted as a control for STB. While research on these individual IPM parameters has been carried out previously, a study which combines all these factors and examines them in an Irish setting is necessary to reach the IPM goal of the Irish NAP.

Whilst this thesis provides a solid contribution to the current body of work on azole sensitivity in *Z. tritici*, this work has also highlighted areas which would undoubtedly benefit from further investigation:

- Repeat the selection experiment (SDHI/azole) in controlled conditions using SDHI lab mutants, or subsequent to SDHI resistance development. That would help provide a more realistic picture of how our treatments would affect less sensitive populations.

- Research on and promotion of IPM for managing STB, i.e. studies on the effects of varietal resistance, seeding date, sowing rate, fertilisation on the development of STB. This would provide up to date information on the benefits of utilising agronomic practices in order to reduce the incidence of disease, and so reducing the pressure on fungicides.
- Inclusion of azole mixtures in modelling studies: to extend the range of models available to cover azole mixtures and alternations, and to validate existing models on combining two high-risk fungicides. Additionally, the inclusion of data from the study of inserts in the promoter could be included into these models.
- An extended study of the *CYP51* gene of the complete collection of isolates in this PhD would be a valuable addition to this work, and the general field of study. It would help explain the observed effects of alternating and reducing doses of azoles. This addition would only be practical if using cost-efficient sequencing methods such as KASP or Next generation genotyping.
- Inserts in the promoter region: while these were associated with reduced sensitivity, further work is needed to ascertain whether it was a causal relationship and if so, what the exact mechanisms were, especially for the ~800 bp insert.
- Further work is needed to elucidate the mechanisms (other than potential over-expression) which cause the large ranges in sensitivities in isolates from a single *CYP51* variant.
- Fitness costs of resistance: to determine whether fitness costs to azole or SDHI insensitivity exist or not.

- A natural progression from this work would be to look at the effects of a full disease control program on sensitivity. Additionally, a study on seed treatments to determine whether azole seed treatments should be included in resistance management plans.

References

Anderson JB, 2005. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. *Nature Reviews Microbiology* **3**, 547-56.

Anon, 2013a. Winter Wheat Recommended list 2013 In. http://www.agriculture.gov.ie/publications/2013/: Department of Agriculture, Food and the Marine. (Accessed: 7/12/14)

Anon, 2013b. Fungicide performance in wheat for 2013. In. http://www.bayercropscience.nl/BAYER/CropScience/BCS_NL.nsf/id/NL_20121213_news_agri_jb_dos/$file/Fungicide_performance_in_wheat_for_2013.pdf: HGCA. (Accessed: 20/12/14)

Anon, 2013c. Sterol Biosynthesis Inhibitor (SBI) Working Group Annual Meeting. In. http://www.frac.info/work/Minutes%20of%20the%202013%20Meeting%20Recommendations%20for%202014.pdf: Fungicide Resistance Action Committee. (Accessed: 28/12/14)

Anon, 2013d. Succinate Dehydrogenase Inhibitor (SDHI) Working Group annual meeting. In. http://www.frac.info/work/Minutes%20of%20the%202013%20SDHI%20Meeting,%20Recommendations%20for%202014.pdf: FRAC. (Accessed: 28/12/14)

Anon, 2014. Fungicide performance in wheat 2014. In. http://cereals.ahdb.org.uk/media/547811/Fungicide-performance-in-wheat-2014-V2.pdf. (Accessed: 28/06/15)

Anon, 2014a. FRAG-UK statement on SDHI fungicides and resistance risk in cereals. In. http://www.pesticides.gov.uk/Resources/CRD/Migrated-Resources/Documents/F/frag-sdhi-statement-cereals-april-2014.pdf: FRAG. (Accessed: 23/12/14)

Anon, 2014b. Fungicide resistance management in cereals. In. http://www.hgca.com/media/175998/frag-2014-fungicide-resistance-management-in-cereals-frag-2014-.pdf: HGCA. (Accessed: 19/12/14)

Anon, 2014c. The HGCA Wheat Disease Management Guide. In. http://www.hgca.com/media/176167/g58-wheat-disease-management-guide-feb-2014-update.pdf: HGCA. (Accessed: 28/06/15)

Anon, 2014d. Succinate Dehydrogenase Inhibitor (SDHI) Working Group annual meeting. In http://www.frac.info/docs/default-source/sdhi-wg/minutes-of-the-2014-sdhi-meeting-recommendations-for-2015-v2.pdf?sfvrsn=4 FRAC. (Accessed 08/07/15)

Anon, 2015. List of species resistant to SDHIs April 2015. In http://www.frac.info/docs/default-source/sdhi-wg/sdhi-references/list-of-species-resistant-to-sdhis-april-2015.pdf?sfvrsn=2: FRAC (Accessed: 08/07/15)

Arenas M, Posada D, 2010. The effect of recombination on the reconstruction of ancestral sequences. *Genetics* **184**, 1133-9.

Arraiano LS, Balaam N, Fenwick PM, *et al.*, 2009. Contributions of disease resistance and escape to the control of Septoria tritici blotch of wheat. *Plant Pathology* **58**, 910-22.

Avenot HF, Michailides TJ, 2010. Progress in understanding moleular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection* **29**, 643-51.

Avenot HF, Sellam A, Karaoglanidis G, Michailides TJ, 2008. Characterization of mutations in the iron-sulphur subunit of succinate dehydrogenase correlating with boscalid resistance in *Alternaria alternata* from California Pistachio. *Phytopathology* **98**, 736-42.

Baccar R, Fournier C, Dornbusch T, Andrieu B, Gouache D, Robert C, 2011. Modelling the effect of wheat canopy architecture as affected by sowing density on *Septoria tritici* epidemics using a coupled epidemic- virtual plant model. *Annals of Botany* **108**, 1179-94.

Bean TP. Amino acid alterations in CYP51 contribute toward reduced triazole sensitivities in a UK field population of *Mycosphaerella graminicola*. *Proceedings of the The BCPC International congress- Crop Science & Technology*, *2005*, 467-70.

Bean TP, 2008. Characterisation of *Mycosphaerella graminicola* isolates with reduced azole sensitivities. In.: HGCA, 1-42.

Bean TP, Cools HJ, Lucas JA, *et al.*, 2009. Sterol content analysis suggests altered eburicol 14α-demethylase (CYP51) activity in isolates of *Mycosphaerella graminicola* adapted to azole fungicide. *Federation of European Microbiological Societies, Microbiology Letter* **296**, 266-73.

Birch CPD, Shaw MW, 1997. When can reduced doses of mixtures delay the build-up of pesticide resistance? A mathematical model. *Journal of Applied Ecology* **34**, 1032-42.

Blake J, Wynn S, Maumene C, Jorgensen LN, 2011. Evaluation of the benefits provided by the azole class of compounds in wheat, and the effect of losing all azoles on wheat and potato production in Denmark, France and the UK. In. *Impact of the loss of all azoles.* ADAS, 23. (Adas, ed.)

Boeger JM, Chen RS, McDonald BA, 1993. Gene flow between geographic populations of *Mycosphaerella graminicola* (Anamorph *Septoria tritici*) detected with restriction length polymorphism markers. *Phytopathology* **83**, 1148-54.

Bolton NJE, Smith JM, 1988. Strategies to combat fungicide resistance in barley powdery mildew. *Brighon Crop Protection conference- Pests and Diseases*, 367-72.

Boukef S, McDonald BA, Yahyaoui A, Rezgui S, Brunner PC, 2012. Frequency of mutations associated with fungicide resistance and population structure of *Mycosphaerella graminicola* in Tunisia. *European Journal of Plant Pathology* **132**, 111-22.

Brent KJ, Hollomon DW, 2007. Fungicide resistance: The assessment of risk. *FRAC monograph No. 2*.

Broscious SC, Frank JA, Frederick JR, 1984. Influence of winter wheat management practices on the severity of powdery mildew and septoria blotch in Pennsylvania. *Phytopathology* **75**, 538-42.

Brown JKM, 2002. Yield penalties of disease resistance in crops. *Current Opinion in Plant Biology* **5**, 339-44.

Brunner PC, Stefanato FL, McDonald BA, 2008. Evolution of the *CYP51* gene in *Mycosphaerella graminicola*: evidence for intragenic recombination and selective replacement. *Molecular Plant Pathology* **9**, 305-16.

Buitrago C, Frey R, Wullschleger J, Sierotzki H. An update on the genetic changes in the *CYP51* gene of *Mycosphaerella graminicola* and thier relationship to DMI fungicide sensitivity. In: Dehne HW, Deising HB, Fraaije B, *et al.,* eds. *Proceedings of the Modern Fungicdes and Antifungal Compounds VII, 2014*. Reinhardsbrunn, Germany: Deutsche Phytomedizinische Gesellschaft, Braunschweig, 103-10.

Burke JJ, Dunne B, 2008. Field testing of six decision support systems for scheduling fungicide applications to control *Mycosphaerella graminicola* on winter wheat crops in Ireland. *Journal of Agricultural Science* **146**, 415-28.

Burnett FJ, Zziwa MCN, 1997. Effect of application rate on the sensitivity of *Erisiphe graminis* f.sp. *tritici* to fenpropimorph. *Pesticide Science* **51**, 335-40.

Chassot C, Hugelshofer U, Sierotzki H, Gisi U. Sensitivity of CYP51 genotypes to DMI fungicides in *Mycosphaerella graminicola*. In: Dehne HW, Deising HB, Gisi U, Kuck HH, Russell PE, Lyr H, eds. *Proceedings of the 15th International Reinhardsbrunn Symposium, 2008*. Friedrichroda, Germany, 129-36.

Christ BJ, Frank JA, 1989. Influence of foliar fungicdes and seed treatments on powdery mildew, septoria and leaf rust epidemics on winter wheat. *Plant Disease* **73**, 148-50.

Clark B, Fraaije B, Lucas J, Cools H, 2010. Septoria resistance and azole use 2010. *RRA newsletter* **January 2010**.

Clark WS, 2006a. Fungicide resistance: are we winning the battle but losing the war? *Aspects of Applied Biology* **78**, 119-26.

Cook RJ, Hims MJ, Vaughan TB, 1999. Effects of fungicide spray timing on winter wheat disease control. *Plant Pathology* **48**, 33-50.

Cools HJ, Bayon C, Atkins S, Lucas JA, Fraaije BA, 2012. Overexpression of the sterol 14α-demethylasegene (*MgCYP51*) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Management Science* **68**, 1034-40.

Cools HJ, Fraaije BA, 2006. Evolution of azole resistance mechanisms in UK populations of *Mycosphaerella graminicola*. *Aspects of Applied Biology* **78**, 21-7.

Cools HJ, Fraaije BA, 2008. Are azole fungicides losing ground against Septoria wheat disease? Resistance mechanisms in *Mycosphaerella graminicola*. *Pest Management Science* **64**, 681-4.

Cools HJ, Fraaije BA, 2013. Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest Management Science* **69**, 150-5.

Cools HJ, Fraaije BA, Lucas JA. Molecular examination of *Septoria tritici* isolates with reduced sensitivities to triazoles. In: Dehne HW, Gisi U, Kuck KH, Russell PE, Lyr H, eds. *Proceedings of the Modern Fungicides and Antifungal Compounds IV, 2005b*. BCPC, Alton, UK, 103-14.

Cools HJ, Fraaije BA, Lucas. JA. Molecular mechanisms correlated with changes in triazole sensitivity in isolates of *Mycosphaerella graminicola*. *Proceedings of the Proceedings of the BCPC congress, Crop Science & Technology, 2005a*. Alton, Hants, UK, 267-74.

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, *et al.*, 2011. Impact of recently emerged sterol 14α-demethylase (CYP51) variants of *Mycosphaerella graminicola* on azole fungicide sensitivity. *Applied and Environmental Microbiology* **77**, 3830-7.

Cools HJ, Parker JE, Kelly DE, Lucas JA, Fraaije BA, Kelly SL, 2010. Heterologous expression of mutated eburicol 14α-Demethylase (CYP51) proteins of *Mycosphaerella graminicola* to assess effects on azole fungicide sensitivity and intrinsic protein function. *Applied and Environmental Microbiology* **76**, 2866-72.

Crawley MJ, 2005. *Statistics: An introduction using R*. The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, England: John Wiley & Sons, Ltd.

Di Tullio E, Camanzi L, Fontolan F, Volpato C, Zucconi S, 2012. The assessment of the economic importance of azoles in European agriculture: Wheat case study. In. Nomisma, Bologna, Italy.

Dinoor A, 1977. Seed treatment of wheat with benzimidazole fungicides against Septoria tritici under semi-arid conditions. *Netherlands Journal of Plant Pathology* **83**, 339-42.

Drabešová J, Ryšánek P, Brunner P, McDonald B, Croll D, 2013. Population genetic structure of *Mycosphaerella graminicola* and Quinone Outside Inhibitor (QoI) resistance in the Czech Republic. *European Journal of Plant Pathology* **135**, 211-24.

Du Rieu AG, Burke RJ, Murray A, 1994. Effectiveness of a novel propiconazole/fenbuconazole mixture for the control of *Septoria* spp. and *Puccinia* spp. in wheat. *Brighton Crop Protection Conference- Pests and Diseases* **1**, 319-24.

Dunne B, Burke JJ, Grace J. Maximising returns from fungicide use in cereals. *Proceedings of the National Tillage Conference, 2008*, 47-58.

Duvivier M, Dedeurwaerder G, De Proft M, Moreau J-M, Legreve A, 2013. Real-time PCR quantification and spatio-temporal distribution of airborne inoculum of *Mycosphaerella graminicola* in Belgium. *European Journal of Plant Pathology* **137**, 325-41.

Estep LK, Torriani SFF, Zala M, *et al.*, 2014. Emergence and early evolution of fungicide resistance in North American populations of *Zymoseptoria tritici*. *Plant Pathology*, n/a-n/a.

Eyal A, 1981. Integrated control of Septoria diseases of wheat. *Plant Disease* **65**, 763-8.

Fillinger S, Omrane S, Sghyer H, *et al.* Characterisation of molecular mechanisms underlying the multi-drug-resistant phenotypes of *Mycosphaerella graminicola* field isolates. In: Dehne HW, Deising HB, Fraaije B, *et al.*, eds. *Proceedings of the Modern Fungicdes and Antifungal Compounds VII, 2014*. Reinhardsbrunn, Germany: Deutsche Phytomedizinische Gesellschaft, Braunschweig, 111-6.

Fraaije B, Cook RJ, Kim SH, Motteram J, Clark WS, Lucas J, 2007. A novel substitution I381V in the sterol 14α-demethylase (CYP51) of *Mycosphaerella graminicola* is differentially selected by azole fungicides. *Molecular Plant Pathology* **8**, 245-54.

Fraaije BA, Bayon C, Atkins S, Cools HJ, Lucas JA, Fraaije MW, 2012. Risk assessments studies on succinate dehydrogenase inhibitors, the new weapons in the battle to control septoria leaf blotch in wheat. *Molecular Plant Pathology* **13**, 263-75.

Fraaije BA, Burnett FJ, Cools HJ, *et al.*, 2011. Understanding evolution and selection of azole resistance mechanisms in UK populations of *Mycosphaerella graminicola*. In. *HGCA Project Report 475*. Home Grown Cereals Authority- HGCA.

Fraaije BA, Cools HJ, Fountaine J, *et al.*, 2005. Role of ascospores in further spread of QoI-resistant cytochrome *b* alleles (G143A) in field populations of *Mycosphaerella graminicola*. *Phytopathology* **95**, 933-41.

Fraaije BA, Lucas JA, Clark WS, Burnett FJ, 2003. QoI resistance development in populations of cereal pathogens in the UK. In. *Proceedings British Crop Protection Council International Congress- Crop Science & Technology*. The British Crop Protection Council, Alton, Hampshire, UK, 689-94. (2.)

Genet J, Jaworska G, Deparis F, 2006. Effect of dose rate and mixtures of fungicides on selection for QoI resistance in populations of *Plasmopara viticola*. *Pest Management Science* **62**, 188-94.

Gigot C, Saint-Jean S, Huber L, *et al.*, 2013. Protective effects of a wheat cultivar mixture against splash-dispersed Septoria tritici blotch epidemics. *Plant Pathology* **62**, 1011-9.

Gladders P, Paveley ND, Barrie IA, *et al.*, 2001. Agronomic and meteorological factors affecting the severity of leaf blotch caused by *Mycosphaerella graminicola* in commercial wheat crops in England. *Annals of Applied Biology* **138**, 301-11.

Gooding MJ, Dimmock JPRE, France J, Jones SA, 2000. Green leaf ares decline of wheat flag leaves: the influence of fungicides and relationships with mean grain weight and grain yield. *Annals of Applied Biology* **136**, 77-84.

Goodwin SB, Ben M'barek S, Dhillon B, *et al.*, 2011. Finished genome of the fungal wheat pathogen *Mycosphaerella graminicola* reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. *PLoS Genetics* **7**, e1002070.

Gosling P, Nicholls C, Watts J, 2014. HGCA Barley disease management guide. In: Boys E, Burgess J, eds. http://www.hgca.com/media/176243/g59-barley-disease-management-guide-feb-2014-update.pdf: HGCA, 28. (Publications H, ed.)

Gough FJ, Lee TS, 1985 Moisture effects on the discharge and survival of conidia of *Septoria tritici*. *Phytopathology* **75**, 180-2.

Graham J, Mcneney B, Seillier-Moiseiwitsch F, 2005. Stepwise detection of recombination breakpoints in sequence alignments. *Bioinformatics* **21**, 589-95.

Green CF, Ivins JD, 1985. Time of sowing and the yield of winter wheat. *Journal of Agricultural Science* **104**, 235-8.

Gressel J, 2010. Low pesticide rates may hasten the evolution of resistance by increasing mutation frequencies. *Pest Management Science* **67**, 253-7.

Griffin MJ, Fisher N, 1985. Laboratory studies of benzimidazole resistance in *Septoria tritici*. *EPPO Bulletin* **15**, 505-11.

Gudmestad NC, Arabiat S, Miller JS, Pasche JS, 2013. Prevalence and impact of SDHI fungicide resistance in *Alternaria solani*. *Plant Disease* **97**, 952-60.

Hall TA, 1999. BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In. Oxford University Press, 95-8. (Nucleic Acids Symposium; vol. 41.)

Hamamoto H, Hasegawa K, Nakaunem R, *et al.*, 2000. Tanden repeat of transcriptional enhancer upstream of the sterol 14α-demethylase gene (*CYP51*) in *Penecillium digitatum*. *Applied and Environmental Microbiology* **66**, 3421-6.

Hawkesford MJ, Araus J-L, Park R, *et al.*, 2013. Prospects of doubling global wheat yields. *Food and Energy Security* **2**, 34-48.

Henze M, Beyer M, Klink H, Verreet JA, 2007. Characterising meteorological scenarios favourable for *Septoria tritici* infections in wheat and estimation of latent periods. *The Americal Phytopathological Society, Plant Disease* **91**, 1445-9.

Hermann D, Gisi U, 1994. Cross-resistance among DMI-fungicides and sensitivity distributions of *Septoria tritici* populations. *Brighton Crop Protection Conference- Pests and Diseases*, 487-92.

Hobbelen PHF, Paveley ND, Fraaije BA, Lucas JA, Van Den Bosch F, 2011a. Derivation and testing of a model to predict selection for fungicide resistance. *Plant Pathology* **60**, 304-13.

Hobbelen PHF, Paveley ND, Oliver RP, 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.

Hobbelen PHF, Paveley ND, Van Den Bosch F, 2011c. Delaying selection for fungicide insensitivity by mixing fungicides at a low and high risk of resistance development: A modelling analysis. *Phytopathology* **101**, 1224-33.

Hobbelen PHF, Paveley ND, Van Den Bosch F, 2014. The emergence of resistance to fungicides. *PLoS One* **9**, e91910.

Hollomon D, Cooke LR, Locke T, 2002. Maintaining the effectiveness of DMI fungicides in cereal disease control strategies. In. *HGCA Project Report 275*. Home Grown Cereals Association- HGCA.

Ishii H, Miyamoto T, Ushio S, Kakishima M, 2011. Lack of cross-resistance to a novel succinate dehydrogenase inhibitor, fluopyram, in highly boscalid-resistant isolates of *Cornespora cassiicola* and *Podosphaera xanthii*. *Pest Management Science* **67**, 474-82.

Jess S, Kildea S, Moody A, Rennick G, Murchie AK, Cooke LR, 2014. European Union policy on pesticides: implications for agriculture in Ireland. *Pest Management Science* **70**, 1646-54.

Kable PF, Jeffery H, 1980. Selection for tolerance in organisms exposed to sprays of biocide mixtures: A theoretical model. *Phytopathology* **70**, 8-12.

Kema GHJ, Verstappen ECP, Todorova M, Waalwijk C, 1996. Successful crosses and molecular tetrad and progeny analyses demonstrate heterothallism in *Mycosphaerella graminicola*. *Current Genetics* **30**, 251-8.

Kendall SJ, Hollomon D, Tolbutt KB, Salter AC, 1996. Triazole mixtures and the control of wheat leaf blotch (*Septoria tritici*). *Brighton Crop Protection Conference- Pests and Diseases*, 251-6.

Kendall SJ, Hollomon DW, 1994. Towards the rational use of triazole mixtures for cereal disease control. *Brighton Crop Protection Conference- Pests and Diseases*, 549-56.

Kildea S, 2009. *Fungicide resistance in the wheat pathogen Mycosphaerella graminicola*. Belfast: The Queen's Universsity of Belfast, PhD.

Kildea S, Glynn L. Cereal disease control. *Proceedings of the National Tillage Conference: Understanding variability to improve precision and profit, 2014*. Kilkenny: Teagasc.

Kildea S, Mullins E, Mercer PC, Cooke LR, Dunne B, O'Sullivan E, 2006. Sensitivity of Mycosphaerella graminicola populations in the Republic of Ireland to DMI and QoI fungicides. *Aspects of Applied Biology* **78**.

Kildea S, Ransbotyn V, Khan MR, Fagan B, Leonard G, Mullins E, Doohan FM, 2008. *Bacillus megaterium* shows potential for the biocontrol of septoria tritici blotch of wheat. *Biological Control* **47:1**, 37-45.

Kildea S, Mehenni-Ciz J, Spink J, O'Sullivan E. Changes in the frequency of Irish *Mycosphaerella graminicola* CYP51 variants 2006-2010. In: Dehne HW, Deising HB, Fraaije B, *et al.*, eds. *Proceedings of the Modern Fungicdes and Antifungal Compounds VII, 2014*. Reinhardsbrunn, Germany: Deutsche Phytomedizinische Gesellschaft, Braunschweig, 143-4.

Koller W, Wilcox WF, 1999. Evaluation of tactics for managing resistance of venturia inaequalis to sterol demethylation inhibitors. *Plant Disease* **83**, 857-63.

Kosman E, Cohen Y, 1996. Procedures for calculating and differentiating synergism and antagonism in action of fungicide mixtures. *Phytopathology* **86**, 1263-72.

Kuck HH, Mehl A, 2004. Prothioconazole: Sensitivity profile and anti-resistance strategy. *Pflanzenschutz-Nachrichten Bayer* **57**, 225-36.

Leitch MH, Jenkins PD, 1995. Influence of nitrogen on the development of Septoria epidemics in winter wheat. *The Journal of Agricultural Science* **124**, 361-8.

Lemey P, Posada D, 2009. Introduction to recombination detection. In: Lemey P, Salemi M, Vandamme A-M, eds. *The Phylogenetic Handbook: A Practical Approach to Phylogenetic Analysis and Hypothesis Testing.* University Printing House, Cambridge, CB2 8BS, United Kingdom: Cambridge University Press, 493-518.

Leroux P, Albertini C, Gautier A, Gredt M, Walker AS, 2007. Mutations in the *CYP51* gene correlated with changes in sensitivity to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* **63**, 688-98.

Leroux P, Gredt M, Walker AS, Moinard JM, Caron D. Resistance of the wheat leaf blotch pathogen *Septoria tritici* to fungicides in France. In: Dehne HW, Gisi U, Kuck KH, Russell PE, Lyr H, eds. *Proceedings of the Modern Fungicides and Antifungal compounds IV, 2005*. BCPC, Alton, UK, 115-24.

Leroux P, Walker AS, 2011. Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* **67**, 44-59.

Levy Y, Levi R, Cohen Y, 1983. Build-up of a pathogen subpopulation resistant to a systemic fungicide under various control strategies: a flexible simulation model. *Phytopathology* **73**, 1475-80.

Librado P, Rozas J, 2009. DnaSP v5: A software for comphrensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451-2.

Linde CC, Zhan J, McDonald BA, 2002. Population structure of *Mycosphaerella graminicola*: from lesions to continents. *Phytopathology* **92**, 946-55.

Luo C-X, Schnabel G, 2008. The cytochrome p450 lanosterol 14α-demethylase gene is a demethylation inhibitor fungicide resistance determinant in *Monilinia fructicola* field isolates from Georgia. *Applied and Environmental Microbiology* **74**, 359-66.

Ma Z, Proffer TJ, Jacobs JL, Sundin GW, 2006. Overexpression of the 14α-demethylase target gene (*CYP51*) mediates fungicides resistance in *Blumeriella jaapii*. *Applied and Environmental Microbiology* **72**, 2581-5.

Mavroeidi VI, Shaw MW, 2005. Sensitivity distributions and cross-resistance patterns of *Mycosphaerella graminicola* to fluquinconazole, prochloraz and azoxystrobin over a period of 9 years. *Crop Protection* **24**, 259-66.

Mavroeidi VI, Shaw MW, 2006. Effects of fungicide dose and mixtures on selection for triazole resistance in *Mycosphaerella graminicola* under field conditions. *Plant Pathology* **55**, 715-25.

Mavroidis VJ, Shaw MW, 2002. Effect of dose rate and mixture on selection for reduced sensitivity to triazole fungicides in *Mycosphaerella graminicola*. *The BCPC Conference-Pests and Diseases* **8D-13**, 859-64.

McDonald BA, 2004. Population genetics of plant pathology. In. *The Plant Health Instructor*. The American Phytopathological Society. Online http://www.apsnet.org/edcenter/advanced/topics/popgenetics/Pages/default.aspx (Accessed 1-5-2015)

Metcalfe RJ, Shaw MW, 1998. Factors affecting the strength of selection for resistance to DMI fungicides in *Septoria tritici*. *Brighton 1998 Conf: Pests Dis. British Crop Protection Council, Farnham, U.K.*, 535-42.

Metcalfe RJ, Shaw MW, Russell PE, 2000. The effect of dose and mobility on the strength of selection for DMI fungicide resistance in inoculated field experiments. *Plant Pathology* **49**, 546-57.

Miedaner T, Zhao Y, Gowda M, *et al.*, 2013. Genetic architecture of resistance to Septoria tritici blotch in European wheat. *BMC Genomics* **14**, 858.

Mikaberidze A, McDonald BA, Bonhoeffer S, 2014. Can high-risk fungicides be used in mixtures without selecting for fungicide resistance? *Phytopathology* **104**, 324-31.

Mullins JGL, Parker JE, Cools HJ, *et al.*, 2011. Molecular Modelling of the Emergence of Azole Resistance in *Mycosphaerella graminicola*. *PLoS One* **6**, e20973.

Nei M, 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA* **70**, 3321-3.

Nikou D, Malandrakis A, Konstantakaki M, Vontas J, Markoglou A, Ziogas B, 2009. Molecular characterization and detection of overexpression C-14 alpha-demethylase-based DMI resistance in *Cercospora beticola* field isolates. *Pesticide Biochemistry and Physiology* **95**, 18-27.

O'Driscoll A, Kildea S, Doohan F, Spink J, Mullins E, 2014. The wheat-*Septoria* conflict: a new front opening up? *Trends in Plant Science* **19**, 602-10.

O'Sullivan E, 2009. Assessing populatuons of the major cereal pathogens for reduced sensitivity to MBC, DMI and Strobilurin fungicides. In. *Teagasc End of Year Report 5078.* Teagasc, 1-16.

O'Sullivan E, Kildea S. Sensitivity of septoria to fungicides- What's new? *Proceedings of the National Tillage Conference, 2010*, 50-60.

Oerke EC, Dehne HW, 2004. Safeguarding production- losses in major crops and the role of crop protection. *Crop Protection* **23**, 275-85.

Oxley S, Gosling P, Nicholls C, Watts J, 2014. HGCA Wheat disease management guide. In: Boys E, Burgess J, eds. http://www.hgca.com/media/176167/g58-wheat-disease-management-guide-feb-2014-update.pdf: HGCA, 28. (Publications H, ed.)

Palmer C-L, Skinner W, 2002. *Mycosphaerella graminicola*: latent infection, crop devestation and genomics. *Molecular Plant Pathology* **3**, 63-70.

Paveley ND, Lockley D, Vaughan TB, Thomas J, Schmidt K, 2000. Predicting effective fungicide doses through observation of leaf emergence. *Plant Pathology* **49**, 748-66.

Paveley ND, Thomas JM, Vaughan TB, Havis ND, Jones DR, 2003. Predicting effective doses for the joint action of two fungicide applications. *Plant Pathology* **52**, 638-47.

Perelló AE, Moreno MV, Mónaco C, Simón MR, Cordo C, 2009. Biological control of Septoria tritici blotch on wheat by Trichoderma spp. under field conditions in Argentina. *BioControl* **54**, 113-22.

Perron GG, Kryazhimskiy S, Rice DP, Buckling A, 2012. Multidrug-therapy and evolution of antibiotic resistance: When order matters. *Applied and Environmental Microbiology* **78**, 6137-42.

Pijls CFN, Shaw MW, 1997. Weak selection by field sprays for flutriafol resistance in *Septoria tritici*. *Plant Pathology* **46**, 247-63.

Ponomarenka A, Goodwin SB, Kema GHJ, 2011. Septoria tritici blotch (STB) of wheat. In. *Plant Health Instructor.*

Quaedvlieg W, Kema GHK, Groenewald JZ, *et al.*, 2011. *Zymoseptoria* gen. nov.:a new genus to accommodate *Septoria*-like species occurring in graminicolous hosts. *Persoonia* **26**, 57-69.

Quérou R, Euvrard M, Gauvrit C, 1998. Uptake and fate of triticonazole applied as seed treatment to spring wheat (*Triticum aestivum* L.). *Pesticide Science* **53**, 324-32.

Russell PE, 2005. A century of fungicide evolution. *Journal of Agricultural Science* **143**, 11-25.

Samoucha T, Gisi U, 1987. Use of two- and three-way mixtures to prevent build-up of resistance to phenylamide fungicides in *Phytophthora* and *Plasmopara*. *The Americal Phytopathological Society* **77**, 1405-9.

Sanders PL, Houser WJ, Parish PJ, Cole J, H., 1985. Reduced-rate fungicide mixtures to delay fungicide resistance and to control selected turfgrass diseases. *Plant Disease* **69**, 939-43.

Scalliet G, Boehler M, Bowler J, Green PS, Kilby PM, Fonne-Pfister R. SDHIs and the fungal succinate dehydrogenase. In: Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE, Lyr H, eds. *Proceedings of the Modern fungicides and Antifungal compounds VI, 2011*. Reinhardsbrunn, Friedrichroda, Germany, 171-8.

Scalliet G, Bowler J, Luksch T, *et al.*, 2012. Mutagenesis and functional studies with succinate dehydrogenase inhibitors in the wheat pathogen *Mycosphaerella gramninicola*. *PLoS One* **7**, e35429.

Schierup MH, Hein J, 2000. Consequences of recombination on traditional phylogenetic analysis. *Genetics* **156**, 879-91.

Schnabel G, Jones AL, 2001. The 14α-demethylase (*CYP51A1*) gene is overexpressed in *Venturia inaequalis* strains resistant to myclobutanil. *Phytopathology* **91**, 102-10.

Schnieder F, Koch G, Jung C, Verreet JA, 2001. Genotypic diversity of the wheat leaf blotch pathogen *Mycosphaerella graminicola* (anamorph) *Septoria tritici* in Germany. *European Journal of Plant Pathology* **107**, 285-90.

Schürch S, Cordette T, 2013. Grundempfindlichkeit der Septoria-Blattdürre des Weizens gegenüber den SDHI-Fungiziden. *Agrarforschung Schweiz* **4**, 82-7.

Shaner G, Finney RE, 1976. Weather and epidemics of septoria leaf blotch of wheat. *Phytopathology* **66**, 781-5.

Shaw MW, 1989. A model of the evolution of polygenically controlled fungicide resistance. *Plant Pathology* **38**, 44-55.

Shaw MW, 1991. Interacting effects of interrupted humid periods and light on infection of wheat leaves by *Mycosphaerella graminicola* (*Septoria tritici*). *Plant Pathology* **40**, 595-607.

Shaw MW, 1993. Theoretical analysis of the effect if interacting activities on the rate of selection for combined resistance to fungicide mixtures. *Crop Protection* **12**, 120-7.

Shaw MW, 2000. Models of the effects of dose heterogeneity and escape on selection pressure for pesticide resistance. *Phytopathology* **90**, 333-9.

Shaw MW, 2007. Is there such a thing as a fungicide resistance strategy? A modeller's perspective. *Aspects of Applied Biology* **78**, 37-43.

Shaw MW, 2009. Fungicide resistance: the dose rate debate. *Outlooks on Pest Management* **20**, 100-3.

Shaw MW, Royle DJ, 1989. Airborne inoculum as a major source of *Septoria tritici* (*Mycosphaerella graminicola*) infections in winter wheat crops in the UK. *Plant Pathology* **38**, 35-43.

Shaw MW, Royle DJ, 1993. Factors determining the severity of epidemics of *Mycosphaerella graminicola* (*Septoria tritici*) on winter wheat in the UK. *Plant Pathology* **42**, 882-99.

Shtienberg D, 1992. Effects of seed treatment with thiabendazole on septoria leaf blotch and growth of wheat. *Plant Disease* **76**, 178-82.

Sierotzki H, Scalliet G, 2013. A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathology* **103**, 880-7.

Simón MR, Cordo CA, Perelló AE, Struik PC, 2003. Influence of nitrogen supply on the susceptibility of wheat to *Septoria tritici*. *Journal of Phytopathology* **151**, 283-9.

Skinner W, Bailey A, Renwick A, Keon J, Gurr S, Hargreaves J, 1998. A single amino-acid substitution in the iron-sulphur protein subunit of succinate dehydrogenase determines resistance to carboxin in *Mycosphaerella graminicola*. *Current Genetics* **34**, 393-8.

Skylakakis G, 1981. Effects of alternating and mixing pesticides on the buildup of fungal resistance. *The Americal Phytopathological Society* **71**, 1119-21.

Stammler G, Carstensen M, Koch A, Semar M, Strobel D, Schlehuber S, 2008. Frequency of different CYP51-haplotypes of *Mycosphaerella graminicola* and their impact on epoxiconazole-sensitivity and -field efficacy. *Crop Protection* **27**, 1448-56.

Stammler G, Glattli A, Koch A, Schlehuber S. Mutations in the target protein conferring resistance to SDHI fungicides. In: Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE, Lyr H, eds. *Proceedings of the Modern fungicides and Antifungal compounds VI, 2011*. Reinhardsbrunn, Friedrichroda, Germany, 195-8.

Stammler G, Semar M, 2011. Sensitivity to *Mycosphaerella graminicola* (anamorph: *Septoria tritici*) to DMI fungicides across Europe and impact on field performance. *EPPO Bulletin* **41**, 149-55.

Stergiopoulos I, Van Nistelrooy JG, Kema GH, De Ward MA, 2003. Multiple mechanisms account for variation in base-line sensitivity to azole fungicides in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* **59** 1333-43.

Strobel D, Bryson R, Stammler G, Semar M. A European overview of the sensitivity of *Mycosphaerella graminicola* (*Zymoseptoria tritici*) to DMI fungicides *in vitro* and the relative impact on field performance. In: Dehne HW, Deising HB, Fraaije B, *et al.*, eds. *Proceedings of the Modern Fungicdes and Antifungal Compounds VII*, *2014*. Reinhardsbrunn, Germany: Deutsche Phytomedizinische Gesellschaft, Braunschweig, 257-62.

Suffert F, Sache I, Lannou C, 2011. Early stages of septoria tritici blotch epidemics of winter wheat: build-up, over seasoning, and release of primary inoculum. *Plant Pathology* **60**, 166-77.

Sundin DR, Bockus WW, Eversmeyer MG, 1999. Triazole treatments suppress spore production by *Puccinia recondita*, *Septoria tritici* and *Stagonospora nodorum* from wheat leaves. *Plant Disease* **83**, 328-32.

Sylvester-Bradley R, Berry PM, Blake J, *et al.*, 2008. *The wheat growth guide*. Caledonian House, 223 Pentonville, London, N1 9HY: HGCA.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, 2013. MEGA6: Molecular Evoultionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725-9.

Thygesen K, Jorgensen LN, Jensen KS, Munk L, 2009. Spatial and temporal impact of fungicide spray strategies on fungicide sensitivity of *Mycosphaerella graminicola* in winter wheat. *European Journal of Plant Pathology* **123**, 435-47.

Tompkins DK, Fowler DB, Wright AT, 1993. Influence of agronomic practices on canopy microclimate and septoria development in no-till winter wheat production in the Parkland region of Saskatchewan. *Canadian Journal of Plant Science* **73**, 331-44.

Van Den Berg F, Van Den Bosch F, Paveley ND, 2013. Optimal fungicide application timings for disease control are also an effective anti-resistance strategy: A Case Study for *Zymoseptoria tritici* (*Mycosphaerella graminicola*) on Wheat. *Phytopathology* **103**, 1209-19.

Van Den Bosch F, Oliver R, Van Den Berg F, Paveley N, 2014a. Governing principles can guide fungicide-resistance management tactics. *Annual Review of Phytopathology* **52**, 175-95.

Van Den Bosch F, Paveley N, Shaw M, Hobbelen P, Oliver R, 2011. The dose rate debate: does the risk of fungicide resistance increase or decrease with dose? *Plant Pathology* **60**, 597-606.

Van Den Bosch F, Paveley N, Van Den Berg F, Hobbelen P, Oliver R, 2014b. Mixtures as a fungicide resistance management tactic. *Phytopathology* **104**, 1264-73.

Veloukas T, Markoglou AN, Karaoglanidis GS, 2013. Differential effect of SdhB gene mutations on the sensitivity to SDHI fungicides in *Botrytis cinerea*. *Plant Disease* **97**, 118-22.

Viljanen-Rollinson SLH, Marroni MV, Butler RC, Deng Y, Armour T, 2005. Latent periods of Septoria tritici blotch on ten cultivars of wheat. *New Zealand Plant Protection* **58**, 256-60.

Walter H. New fungicides and new modes of action. In: Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE, Lyr H, eds. *Proceedings of the Modern fungicides and Antifungal compounds VI, 2011*. Reinhardsbrunn, Friedrichroda, Germany, 47-54.

Whittenberg AHJ, Van Der Lee TaJ, Ben M'barek S, *et al.*, 2009. Meiosis drives extraordinary genome plasticity in the haploid fungal plant pathogen *Mycosphaerella graminicola*. *PLoS One* **4**, e5863.

Yoshida Y, Aoyama Y, 1987. Interaction of azole antifungal agents with cypochrome P-450_{14dm} purified from *Saccharomyces cerevisiae* microsomes. *Biochemical Pharmacology* **36**, 229-35.

Zadocks JC, Chang TT, Konzak CF, 1974. A decimal code for the growth stages. *Weed Research* **14**, 415-21.

Zhan J, McDonald BA, 2004. The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola*. *Fungal Genetics and Biology* **41**, 590-9.

Zhan J, Mundt CC, McDonald BA, 1998. Measuring immigration and sexual reproduction in field populations of *Mycosphaerella graminicola*. *Phytopathology* **88**, 1330-7.

Zhan J, Mundt CC, McDonald BA, 2007. Sexual reproduction facilitates the adaption of parasites to antagonistic host environments: evidence from empirical study in the wheat-*Mycosphaerella graminicola* system. *International Journal of Parasitology* **37** 861-70.

Zhan J, Pettway RE, McDonald BA, 2003. The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. *Fungal Genetics and Biology* **38**, 286-97.

Zhan J, Stefanato FL, McDonald BA, 2006. Selection for increased cypoconazole tolerance in *Mycosphaerella graminicola* through local adaption and in response to host resistance. *Molecular Plant Pathology* **7**, 259-68.

Zwiers L-H, Stergiopoulous I, Van Nistelrooy JGM, De Waard MA, 2002. ABC transporters and azole sensitivity in laboratory strains of the wheat pathogen *Mycosphaerella graminicola*. *Antimicrobial Agents and Chemotherapy* **46**, 3900-6.

Zziwa MCN, Burnett FJ, 1994. The effect of reduced doses on the sensitivity of powdery mildew to fenpropimorph in barley field trials. *BCPC Monograph No 60: Fungicide resistance*, 303-8.