University of Reading

The status of fungicide resistance in South American *Zymoseptoria tritici* populations

Thesis submitted for the degree of Doctor of Philosophy

School of Agriculture, Policy and Development, University of Reading

Department of Biointeractions and Crop Protection, Rothamsted Research

Guilherme Rossato Augusti

December 2019

Abstract

Septoria tritici blotch, causal agent *Zymoseptoria tritici*, is one of the most important wheat diseases and has a worldwide distribution. Fungicides are essential for the maintenance of reliable wheat yields in areas under high incidence of *Z. tritici*. Most modern fungicides have single-site modes of action (MoA). Prolonged use of the same MoA to control the pathogen population leads to the selection of isolates with reduced sensitivity. The emergence of resistance to these fungicides can, therefore, compromise disease control and reduce yields.

This research aimed to determine whether South American *Z. tritici* field populations are evolving reduced fungicide sensitivity and to characterize underlying resistance mechanisms in comparison to worldwide field populations of *Z. tritici*. The results from *in vitro* sensitivity assays indicate that there is reduced sensitivity to methyl benzimidazole carbamate (MBC), azoles, quinone outside inhibitor (QoI) and succinate dehydrogenase inhibitor (SDHI) fungicides in South American isolates of *Z. tritici*. Isolates with highly resistant MBC and QoI phenotypes carried the target site amino acid substitutions E198A and G143A, respectively. Target site mutations conferring SDHI resistance were not identified, but some isolates had fluopyram resistance phenotypes possible associated with the presence of a previously-reported additional paralogue of succinate dehydrogenase subunit C in worldwide populations of *Z. tritici*. Reduced azole sensitivity was conferred by different combinations of amino acid substitutions in the target site sterol 14α-demethylase (CYP51). Population genetic analyses with microsatellite markers and sequence data of the azole target site identified that the European CYP51 encoding gene has introgressed into South American populations of *Z. tritici*.

The results of this research indicate that South American populations of *Z. tritici* have the potential to evolve levels of fungicide resistance similar to those observed in Europe, which could compromise the future management of Septoria tritici blotch in South America.

Declaration

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

Guilherme Rossato Augusti

Acknowledgements

I wish to thank the Brazilian Federal Foundation for Support and Evaluation of Graduate Education (CAPES, Portuguese acronym) for funding my PhD programme.

I would also like to thank many people who supported me throughout this PhD:

My supervisor, Dr Bart A. Fraaije, and my co-supervisor, Dr Nichola Hawkins, for welcoming me into the Fungicide Research Group at Rothamsted Research and for all the teaching and training for the last four years.

Professor Michael Shaw, at the University of Reading, for all his guidance, advice and ideas for my research project and his support during the writing of this thesis.

My colleagues and friends at Rothamsted Research.

The various collaborators who helped me by providing infected wheat leaf samples: Gladys Clemente (INTA - Argentina); Ángela Norma Formento (INTA - Argentina); María Rosa Simón (Universidad Nacional de La Plata); Silvia A. Pereyra (INIA - Uruguay); Christina H. Hagerty (Oregon State University); Gregory Kemmitt and Pablo Riveros Calderon (Corteva Agriscience); Pavel Matušinsky (The Agricultural Research Institute Kromeriz); Božena Sedláková (Palacky University in Olomouc); Martin Pastircak (Plant Production Research Institute in Piešťany); Mikaela Breunig (Michigan State University); and Rachael Warren (The New Zealand Institute for Plant and Food Research).

Finally, to my family and my girlfriend, Cristine, for being so supportive and keeping me going over the last four years. Thank you.

Table of Contents

List of Figure

List of Tables

Abbreviations

ABC: ATP-Binding Cassette

a.i.: active ingredient

ANOVA: Analysis of variance

AMOVA: Analysis of molecular variance

ATP: Adenosine TriPhosphate

bp: base pair

DMI: Demethylation Inhibitor

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

dNTP: Deoxynucleotide Triphosphates

EC_{50}: 50% Effective Concentration

EDTA: Ethylenediaminetetraacetic acid

FRAC: Fungicide Resistance Action Committee

FRAG-UK: Fungicide Resistance Action Group

IPM: Integrated Pest Management

MBC: Methyl Benzimidazole Carbamate

MDR: Multi-Drug Resistance

MFS: Major Facilitator Superfamily

ML: Maximum Likelihood

MoA: Mode of Action

PCR: Polymerase Chain Reaction

QiI: Quinone inside Inhibitor

QoI: Quinone outside Inhibitor

SDB: Sabouraud Dextrose Broth

SDHI: Succinate Dehydrogenase Inhibitor

STB: Septoria tritici Blotch

YPD: Yeast extract Peptone and Dextrose agar

Chapter 1: Introduction

1.1 *Zymoseptoria tritici*

1.1.1 *Zymoseptoria tritici* biology

Wheat is one of the most important crops for global food security. It provides 19% of the daily caloric needs and 21% of the daily dietary protein intake (Shiferaw *et al.*, 2013). Wheat is grown in more land than any other crop in the world and is cultivated over a wide range of climatic conditions. According to FAO's biannual Food Outlook report (FAO, 2019), the global production of wheat in 2018/19 was estimated to be around 730.2 million tonnes.

The wheat pathogen *Zymoseptoria tritici* (formerly *Septoria tritici*), the causal agent of Septoria tritici blotch (STB), has a great impact on the worldwide wheat production. STB is the most important foliar wheat disease in Europe, one of the three most devastating wheat foliar diseases in North America, and can cause significant yield losses in South America. Reductions in wheat yield by 35 to 50% have been reported as a result of severe epidemics of STB (Ponomarenko *et al.*, 2011). A recent study estimated that the return in controlling STB for farmers in Europe can be 2,5 to 7 fold the investment made with chemical control (Fones & Gurr, 2015).

According to Eyal *et al.*, (1987) *Septoria tritici* Rob. Ex Desm., was first reported on wheat by Desmazieres in 1842. The sexual stage (teleomorph) *Mycosphaerella graminicola* (Fuckel) J. Schrot. in Cohn, was described by Sanderson in 1972 in wheat stubble collected from a field in New Zealand (Sanderson, 1972). In 2011, Quaedvlieg *et al.* introduced the new genus *Zymoseptoria* to accommodate the *Septoria*-like species occurring in graminicolous hosts, and since then the causal agent of Septoria tritici blotch in wheat is named *Zymoseptoria tritici* (Quaedvlieg *et al.*, 2011).

Stukenbrock *et al.* (2006) and Banke *et al.* (2004) concluded that the wheat-adapted *Z. tritici* originated in the ancient Fertile Crescent, around 10,500 years ago, coevolving as a pathogen of the native populations of wheat since its divergence from the ancestral pathogen that infected wild grasses. With

the process of domestication of wheat, the pathogen has been “co-domesticated” and spread further with its host, firstly to Europe and Asia and later to the Americas and Australia.

The asexual stage of *Z. tritici* causes most of the symptoms developed throughout the season on wheat plants. Symptoms are characterized by chlorotic blotches on leaves, starting on first leaves at early growth stages. When the chlorotic spots become necrotic lesions the pycnidia (fruiting bodies) can be found as dark spots, on both sides of the leaves. The pycnidia are the asexual reproductive structures developed within the substomatal cavities and vary in size (100-200 µm in diameter) (Steinberg, 2015) depending on the cultivar-strain interaction and on the infection density (Eyal *et al.*, 1987). Conidia termed pycnidiospores (macropycnidiospores and micropycnidiospores) are formed within pycnidia (Eyal *et al.*, 1987). The macropycnidiospores are multi-cellular structures, consisted of 4 to 8 cells measuring ~1.5-3.54 µm wide and up to ~40-100 µm long each (Steinberg, 2015). These structures are also formed when the fungus is grown under laboratory conditions, known as the yeast-like stage, and its dimensions vary between field isolates (Steinberg, 2015). The micropycnidiospores are uni-cellular structures, measuring ~1 µm wide and ~5-10 µm long, formed by yeast-like budding from the macropycnidiospores or hyphal cells. The pycnidia exude masses of pycnidiospores (cirrhi) when mature (Wiese, 1987). Both types of pycnidiospores can infect wheat. Once the macropycnidiospores germinate they form a hypha that extends by polar tip growth (Steinberg, 2015).

The teleomorph, *Mycosphaerella graminicola*, can be found as sexual fruiting bodies, termed perithecia, on senescent leaves and wheat stubble. Perithecia take twice as long to develop than pycnidia that can be seen 3-4 weeks after infection. These structures are subepidermal, dark and measure 48 x 114 µm in diameter (Wiese, 1987). They are formed due to heterothallism, which is the encounter of two strains of opposite mating types (Kema, 1996), termed MAT1-1 and MAT1-2. Perithecia form asci, 11-14 by 30-40 µm in size, containing eight ascospores each. The ascospores are hyaline, elliptical, 2-3 by 10-15 µm in size, and consist of two cells (Wiese, 1987).

1.1.2 Epidemiology

The airborne sexually produced ascospores of the teleomorph *Mycosphaerella graminicola* are the main source for the primary infections (Shaw & Royle, 1989, 1993), whereas the asexual pycnidiospores of *Z. tritici* are the main source of secondary infections. Infected straw, seed, and volunteer wheat can also be sources of primary inoculum, while non-wheat hosts seem not seem to play an important role in disease epidemics (Wiese, 1987). Asexual pycnidiospores are produced during wet periods and disseminated from leaf to leaf by rain splash over the canopy (Wiese, 1987).

Steinberg (2015) divides the infection process into the latent phase (biotrophic phase) and necrotrophic phase. The infection begins with the germination of the pycnidiospores or ascospores, forming thin germ tubes terminally or laterally from the central part of spores (Shetty *et al.*, 2003). Wiese (1987) cites that spore germination occurs after six hours of wetness with temperatures between 15 and 25°C, but can also occur between five and 35°C. After contact with the leaf, spores switch from yeast-like to hyphal growth and the hyphae enter the tissue via stomata (Kema, 1996). Unlike some other species of fungi (Allen, 1991), *Z. tritici* does not require an appressorium to penetrate the host, and thigmotropic or chemotropic signals are not important for successful penetration (Kema, 1996). Moreover, Fones et al. (2017) found that *Z. tritici* is capable of random epiphytic growth on susceptible wheat leaves, remaining on the leaf surface for ten days or longer. However, Duncan & Howard (2000) suggested that germ tubes grow towards stomata, in response to a signal, and believe that the fungus produces an appressorium-alike structure. Shetty et al. (2003) confirmed that penetration through the stomata always took place from germ tubes that produced appressorium-alike swellings.

The fungus requires a period of at least 48 h to reach the mesophyll cells and start the colonization, where the hyphae remain exclusively in the intercellular space (Kema, 1996), branching laterally and forming a dense network within the first 9-15 days after infection (Shetty *et al.*, 2003). During this period fungus biomass is barely detectable due to the lack of nutrients (Steinberg, 2015). About 9-11

days after infection, hyphae aggregate and pre-pycnidia appear in the substomatal cavities, being fully completed around 11-15 days after the infection begins (Duncan & Howard, 2000; Shetty *et al.*, 2003). Until this moment the infection remained asymptomatic; the so-called latent period or biotrophic phase. Because *Z. tritici* does not form haustoria or any other clear feeding structure (Kema, 1996) it seems the fungus can survive on its stored nutrient reserves (Sánchez-Vallet *et al.*, 2015).

The pycnidia are formed from extensive growth of hyphae that invade the substomatal cavity. Once the asexual fruiting bodies are formed the fungus switches to the necrotrophic phase, which is characterized by the disintegration of the host tissue (Kema, 1996), resulting in the appearance of chlorotic blotches, progressing to necrotic areas. The symptoms appear 14 to 21 days after infection begins, depending on cultivar susceptibility, fungal isolate and environmental conditions (Eyal *et al.*, 1987). Kema et al. (1996) found that the pycnidium formation only completes after cell collapse because this provides the nutrients to increase fungal biomass.

The macropycnidiospores produced in the pycnidia will be rain-splashed through the leaf canopy. After 30 minutes of leaf wetness, pycnidiospores can be released from the top of the pycnidium, through the ostiole, in the form of an oozing drop called a cirrhus (Eyal *et al.*, 1987). *Z. tritici* can liberate between $5x10^3$ and $10x10^3$ pycnidiospores from each pycnidium (Eyal, 1971).

1.1.3 Specificity and host resistance

Eyal et al. (1973) first reported physiological specialization in the *Z. tritici*-wheat pathosystem. Specific interactions were confirmed, and Brading et al. (2002) showed that a gene-for-gene relationship is also applicable for the wheat-*Z. tritici* pathosystem. So, for every gene conferring resistance in the host, there is a corresponding gene for avirulence in the pathogen (Flor, 1971). It means that major resistance genes can be overcome if the pathogen loses or alters its virulence gene, as happens for other diseases with the gene-for-gene system. Recently, AvrStb6 was identified as an avirulence gene in *Z. tritici* isolates with avirulence phenotype on wheat cultivars carrying the Stb6 resistance gene (Zhong *et al.*, 2017; Kema *et al.*, 2018; Saintenac *et al.*, 2018).

Breeding wheat for *Z. tritici* resistance is not easy. The pathogen population size is large and because the fungus can reproduce both sexually and asexually, populations have high genetic diversity, and significant gene flow can be measured in wheat-growing regions within and between seasons (Zhan & McDonald, 2004). Due to these characteristics, the pathogen can easily overcome plant resistance as well as evolving fungicide resistance. Breeding for host resistance based on major genes with a large effect, termed qualitative resistance, is not durable. One example of overcoming a major gene resistance was seen with cultivar Gene in Oregon, which became susceptible 5 years after its release (Brading *et al.*, 2002).

Quantitative resistance, which is controlled by several genes with each contributing moderate effects, can be more effective for STB control. Indeed, resistance to STB in field trials generally appears as a quantitative trait, and Brown et al. (2015) reviewed the literature and conclude that, until 2015, 89 regions of the wheat genome had been reported carrying quantitative trait loci (QTL) or meta-QTL for resistance to STB. This type of resistance is more durable than the qualitative resistance, and although can be eroded, it happens slowly over the years. Hence, quantitative resistance to this pathogen can be a promising tool for STB control, especially when combined with chemical control. Since the 1970s, Ziv and Eyal (1977) have already demonstrated resistance to STB in some wheat cultivars, although, it seems that the progress in breeding new varieties for resistance has been slow since then (Parker *et al.*, 2004).

1.1.4 Diversity

Z. tritici populations have high levels of variability. Even a single lesion can be colonized by several genetically different individuals, partially due to the presence of both asexual and sexual reproduction (Boeger *et al.*, 1993). The fact that different genotypes colonize the same lesion allows an increase in recombination because there is a higher chance that isolates of different matting types find each other. A survey of the mating-type locus, encoded by either *MAT-1* or *MAT-2* (Zhan *et al.*, 2002) showed that both mating types coexist at an approximately equal frequency at all spatial scales analysed, from

lesions to continents, indicating the sexual stages occurs frequently. Zhan *et al*. (2003) indicate that populations of *Z. tritici* have a high level of diversity in the nuclear genome, characterized by high gene diversity (frequency of alleles at individual loci) and high level of genotype diversity (frequency of genetically distinct individuals in the population). McDonald *et al.* (1995) found a large genetic variation on small scales (within field populations) but a high degree of genetic similarity among populations geographically separate within a country, probably because there was substantial gene flow between regions. Studying isolates from two sub-regions of the Argentinean province of Buenos Aires, Castillo et al. (2010) detected a high degree of diversity in the *Z. tritici* population in Argentina, and high gene flow between both sub-regions, indicating the importance of sexual ascospores as a source of genetic exchange between distant populations. Zhan and McDonald (2004) proved that gene flow is one of the major evolutionary force homogenising the genetic structure of local *Z. tritici* populations within regions.

Simón et al. (2012) attribute the differences in the genetic variability of *Z. tritici* populations around the world to different migration patterns, variation in recombination and the importance of the sexual form. Sexual reproduction plays a major impact on the genetic structure of populations of *Z. tritici* compared to asexual reproduction, and airborne movement of ascospores can be responsible for gene flow for a few hundreds of kilometres (McDonald *et al.*, 1995). Linde *et al*. (2002) provided evidence that gene flow in *Z. tritici* within regions is much higher than among continents, but their study concludes that populations on different continents were genetically similar. As they explain, it is unlike ascospores flying such long distances to be responsible for genetic exchange, and so human movement of plant material infected with mycelium or pycnidiospores is responsible for intercontinental dissemination. Data from populations around the world showed that they have a similar genetic structure and a high degree of similarity (McDonald *et al.*, 1995). So, it appears that most genetic variation is distributed at a single field scale, where a population contains many genotypes, but populations separated over long distances are genetically similar. Zhan *et al*. (2003) conducted a global assay to analyse the spatial structure of *Z. tritici* and found that ~80% of the genetic diversity was

distributed within field sites measuring 1-9 m^2, ~12% of diversity was distributed among sites within a field, ~5% was distributed among fields within a region and only ~3% among different continents.

The centre of origin for *Z. tritici* is hypothesized to be the Fertile Crescent (Stukenbrock *et al.*, 2006), and so genetic diversity is expected to be higher in populations from the Israel region than other locations around the world (Linde *et al.*, 2002). It was proved that populations from Israel have a higher degree of variation in the mtDNA and nuclear DNA than other populations studied (McDonald *et al.*, 1995). This hypothesis is also supported by Abrinbana *et al*. (2010), who revealed intermediate to high levels of genetic diversity in populations of *Z. tritici* from five wheat-growing regions in Iran. In a global survey across continents, Banke and McDonald (2005) described that the Israeli *Z. tritici* population had many unique haplotypes not found in other populations. *Z. tritici* has great genetic variation, that associated with the regular sexual reproduction and large population size, enabling the pathogen to easily generate new allele recombination, while the asexual reproduction assures rapid local selection of new virulence and/or fungicide resistance alleles due to high overproduction of offspring (Zhan & McDonald, 2004).

1.1.5 Importance of STB in South America

Although the status and mechanisms of fungicide resistance are well known in European populations of *Z. tritici*, this subject has not been explored with South American populations, where *Z. tritici* is an important wheat pathogen in areas with high humidity and temperate climates. The first wheat yield losses due to STB in South America have been reported back in 1939 in Argentina and Uruguay (Boerger, 1943) and in 1975-76 in Chile (Gilchrist & Madariaga, 1980).

Wheat is the most important winter crop in Argentina, where it has been cultivated for more than 100 years. In the 2018/2019 growing season, Argentina produced 19,459,727 t of wheat from 6,050,953 ha (MAGYP, 2019). The traditional Argentinian wheat-growing region encompasses the provinces of Santa Fe, Córdoba, La Pampa, Entre Ríos and Buenos Aires (Stocco, 2014). STB is an endemic disease in Argentina, being the most important foliar disease in the southeast region (Kraan & Nisi, 1993), where

the Buenos Aires province is located. The incidence of STB is higher in years of low temperatures and high humidity when the pathogen can be found in wheat leaves at the beginning of tillering, but it is more common to be found around the flowering period (Kraan & Nisi, 1993). Yield losses from 16 to 50% have been reported in Argentina due to STB (Kraan & Nisi, 1993; Simón *et al.*, 2002, 2012). Since host resistance is not efficient enough, STB control relies on programmed fungicides applications. Fungicide applications have shown to be profitable for growers with yield increases between 20 and 32%, and, as a consequence, their usage has increased yearly since 1996 (Cordo, 2010). Mixtures of triazoles and strobilurins have been used to control STB, and most recently the SDHIs have also entered the market. The use of fungicides on wheat is required in Argentina to control rusts and/or leaf spots. *Puccinia triticina* causal agent of leaf rust or orange rust is currently the main wheat disease in Argentina, and therefore one of the main reasons for the use of fungicides in the crop. Mixtures of azoles, strobilurins, and SDHIs are the most effective combinations of fungicides to control this rust (Fleitas *et al.*, 2016). Yellow rust (*Puccinia striiformis* f.sp. *tritici*) caused significative yield losses in the 2017 wheat season and fungicides spray is required for susceptible cultivars (INTA - Argentina, 2018). Wheat stem rust (*Puccinia graminis* f.sp. *tritici*), has also gained importance due to the adoption of susceptible varieties and an increase in the temperature levels towards the end of the crop cycle (Germán et al., 2007). Besides STB, tan spot (*Drechslera tritici-repentis*) is the other leaf spot controlled with fungicides (Moreno & Perelló, 2010), for which QoI resistant strains are present in Argentina (News Room, 2019). The intense use of fungicides to control rusts and other fungal wheat diseases in Argentina might also increase the fungicide selection pressure on *Z. tritici* even if it is not the target of chemical sprays.

In Chile, 222,705 ha were sown with wheat in the 2018/2019 growing season, and 1,399,919 t of grain was harvested (ODEPA - Oficina de Estudios y Políticas Agrarias, 2019), indicating the high yield potential of wheat production of Chile when compared to others countries in South America. Septoria tritici blotch is one of the most important wheat diseases, being present in all the wheat-growing regions of the country (Andrade & Contreras, 2007). The yield losses reported vary from 2 to 40%

(Caglevic, 1982; Madariaga B. & Scharen, 1985; Andrade & Contreras, 2007), depending on the region and the rainfall. Early sowing wheat in cold and wet winter that will complete its cycle in temperate humid spring are the ideal conditions for STB development. STB progress tends to decrease considerably from July and disappear in September (Mellado, 1990). In the Centre-South Chile region, early-sow of susceptible spring wheat varieties in critical months of April to May had significant yield losses due to STB and the protection of the flag leaf with one fungicide spray was profitable (Mellado, 1990). Chemical control of STB in Chile is by multi-site fungicides, methyl benzimidazole carbamates (MBCs) and mainly triazole-strobilurin mixtures (Andrade & Contreras, 2007), with a reported increase in yield production of up to 32.8% compared with yields of untreated plots (Madariaga, 2011). More recently the new SDHIs compounds have also been introduced for STB control. *Z. tritici* may be selected for fungicide resistance even when it is not the target pathogen in the field. In the Araucania region, other wheat diseases need to be controlled with fungicide applications, such as *Drechslera tritici repentis*, *Puccinia striiformis*, *Puccinia triticina* and *Blumeria graminis* (Galdames, 2013). However, these have a late incidence in the field and generally require fungicide applications only to protect the flag leaf or ears that consequently tend to be sprayed when *Z. tritici* may have reduced its progress on the field due to climate conditions.

The Uruguayan wheat production in the 2018/2019 season was 728,307 t harvested from 198,000 ha (OPYPA - Oficina de Programación y Políticas Agropecuarias, 2019). After many observations, between 1967 and 2002, Ackermann (2010) reported yield losses of 30% in Uruguayan wheat crop due to STB, besides the grain quality reduction in years of high disease pressure. Fungicide spraying is the main strategy for STB control to achieve high wheat production, and in Uruguay, MBCs, DMIs and QoIs have been used for a long time with this purpose (Ackermann, 2010). Reduction in control effectiveness has already been described for benomyl (Ackermann, 2010), indicating that MBC resistant isolates may already be present at high frequencies in Uruguayan populations of *Z. tritici*. In INIA La Estanzuela (Colonia-Uruguay) field experiments have been carried out between 2004 and 2006 for evaluation of STB control with fungicides, and data analysis shows the highest efficacy of disease control is achieved

using mixtures of azoles and strobilurins (Ackermann, 2010). As in Argentina and Chile, other wheat leaf diseases also rely on chemical control in Uruguay (INIA - Uruguay, 2017). Leaf rust (*Puccinia triticina*) is currently one of the most significant wheat diseases in Uruguay and under severe epidemics, two or more fungicide applications are necessary to control the disease in susceptible cultivars (Scholz *et al*., 2019). *Pyrenophora tritici-repentis*, the causal agent of Tan spot, is also present in Uruguay and fungicide is often deployed to prevent disease-causing significative yield losses (Olivet *et al.*, 2017).

1.2 Mode of action of fungicides

1.2.1 Methyl Benzimidazole Carbamates

The introduction of methyl benzimidazole carbamates (MBCs) fungicides in the late 1960s was the launch of a new era in chemical control of plant pathogens, with new compounds effective at low doses, targeting a broad spectrum of fungi and, most importantly, with systemic translocation in the host plant, providing control of pathogens even after infection (Delp, 1995). The methyl benzimidazole carbamates are subdivided into two chemical groups, the benzimidazoles and the thiophanates (FRAC, 2017), and although thiophanates are not benzimidazoles until transformed, they are referred to as benzimidazoles.

All benzimidazole fungicides interfere with microtubule assembly because they bind to the β-tubulin subunit of tubulin, the major component of microtubules. Because microtubules are the cytoskeletal polymers in eukaryotic cells, benzimidazole fungicides block fungal meiosis and mitosis, arresting nuclear division. The specificity of benzimidazole fungicides is guided by its chemical structure, when selected they should not bind to mammalian tubulin (Ishii, 1992; Davidse & Ishii, 1995).

1.2.2 Demethylation inhibitor fungicides

Fungicide compounds acting on the biosynthesis of sterol were first synthesised during the second half of the 1960s. Some of these compounds inhibit the demethylation at position 14 of lanosterol, the precursor of sterol in fungi, and so are grouped as demethylation inhibitors (DMIs) (Kuck *et al.*, 1995).

Imidazoles and triazoles, also designated together as azoles, are the most important groups of DMI fungicides. DMIs are highly potent antifungal compounds and the largest group between the sterol biosynthesis inhibiting (SBIs) fungicides, which include the amines (previous called morpholines), keto-reductase inhibitors and the squalene-epoxidase fungicide groups. All DMIs can penetrate the plant cuticle and translocate via the apoplast, with minimal or no symplastic translocation. This systemic property improves the control of plant diseases when the pathogen is already established, as a curative treatment, and the uptake of the fungicide into the plant allows better coverage of the chemical treatment (Kuck *et al.*, 1995).

In filamentous fungi, ergosterol is produced by the lanosterol sterol biosynthesis pathway. The sterol 14α-demethylase (known as CYP51), an enzyme of the cytochrome P-450 superfamily, is responsible for an important step in this sterol biosynthesis process, the demethylation of lanosterol (Koller, 1992). The DMI fungicides bind the haem iron of the 14α-demethylase due to a nitrogen heterocycle with at least one unhindered N atom acting as a ligand to the haem iron. This causes the inhibition of the biding of either the substrate or of molecular oxygen to the catalytic site of the cytochrome P-450, resulting in accumulation of the enzyme substrate (eburicol or lanosterol) and depletion of ergosterol, an important membrane component, disrupting the structure of plasma membrane (Kwok, 1993; Berg & Plempel, 1998).

1.2.3 Quinone outside inhibitor fungicides

Introduced into the market in 1996 the quinone outside inhibitor fungicides (QoIs), also known as strobilurins, are an important class of agricultural fungicides (Bartlett *et al.*, 2002). They were discovered as natural fungicidal compounds, produced by the basidiomycete wood-rotting fungi *Oudemansiella mucida* (Schrad ex Fr) Hoehn and *Strobilurus tenacellus* (Pers ex Fr), with a capacity to inhibit microbial respiration at cytochrome bc_1 (Complex III inhibitors) (Bartlett *et al.*, 2002; Yamaguchi & Fujimura, 2005).

The fungal mitochondrial respiration pathway involves three proton translocating complexes (I, III and IV), linked by ubiquinone and cytochrome *c* as mobile electron carriers to provide energy production with ATP synthesis. In complex III, a cytochrome *b* with two ubiquinone-binding sites (Qo and Qi) is part of the cytochrome bc_1 complex, located in the inner mitochondrial membrane. The QoIs bind to the Qo site of cytochrome *b*, thereby blocking the electron transfer between cytochrome *b* and cytochrome c_1, and consequently affecting the energy cycle (Bartlett *et al.*, 2002; Yamaguchi & Fujimura, 2005). QoI fungicides have shown to have excellent efficacy to control a broad-spectrum of plant diseases and can also have some curative properties. The systemic properties vary between the different compounds, and whilst some of them can move to the tip of leaves, or even to newly expanding and newly emerging growth plant tissue, others showed no such systemic movement (Bartlett *et al.*, 2002).

1.2.4 Succinate dehydrogenase inhibitor fungicides

The first representatives of succinate dehydrogenase inhibitors (SDHIs), also known as carboxamides, were carboxin and oxycarboxin, reported in 1966, with main activity against diseases caused by basidiomycetes (White & Georgopoulos, 1992). Recently, new chemical groups with the same mode of action of the old carboxamides, but with improved design regarding chemical structure, have been launched to target a wide range of pathogens on various crops. SDHI fungicides are inhibitors of the mitochondrial succinate dehydrogenase (SDH), also known as complex II of the mitochondrial respiration chain, a functional part of the tricarboxylic cycle and mitochondrial electron transport chain (White & Georgopoulos, 1992; Schewe & Lyr, 1995).

The succinate dehydrogenase is a complex of four subunits, the hydrophilic flavoprotein (A) and iron-sulfur protein (B) and two lipophilic transmembrane C- and D-subunits. It is anchored in the inner mitochondrial membrane. SDH links the carboxylic acid cycle and cellular respiration because it catalyses the oxidation of succinate to fumarate and reduces ubiquinone to ubiquinol. SDHIs bind to the ubiquinone-binding site of the mitochondrial complex II blocking electron transport to ubiquinone, preventing its reduction and overall respiration, and, therefore, affecting the energy production within

cells. This class of fungicides is also known by its systemic movement took up by the cuticle and translocated throughout plant tissues via the xylem system (White & Georgopoulos, 1992; Schewe & Lyr, 1995; Stammler *et al.*, 2007; Dehne *et al.*, 2010; Avenot & Michailides, 2010).

1.3 Mechanisms of fungicide resistance

Crop yield losses due to the attack of plant pathogens are a threat to food security. In the absence of host resistance, fungicides are the main method to control fungal pathogens that causes diseases on plants. Since the introduction of the fungicide compounds with specific modes of action and systemic mobility, during the 1960s, their continuous use on crops has selected resistant strains of different plant pathogens, reducing their efficiency for disease control. Delp (1988) reports that until 1970, due to the predominant use of multisite fungicides, less than 10 genera of fungi showed field resistance; but with the increase of the systemic fungicides, this number had increased to nearly 60 genera in 1988. The risk of developing resistance depends on the chemical group to which the fungicides belong, and, even inside the same fungicide class, differences in the molecular structures can influence the resistance risk, as with the compounds in the DMIs class. Fungicides belonging to the same chemical group often show cross-resistance. This means that resistant strains to one fungicide are also resistant to other fungicides with the same mode of action. The way that resistance to fungicides is generated, spread, and maintained in fungal populations is not fully understood, but so far, many mechanisms responsible for fungicide resistance in *Z. tritici* have been elucidated and are discussed below, especially those to DMIs.

1.3.1 Alteration in the target protein

Alteration of the target site is the most common way in which fungi can become resistant to a fungicide. Modern single-site fungicides target specific proteins to which they bind and block a specific biochemical process in a spore or mycelium. An alteration in the three-dimensional structure of the target site can prevent the fungicide from binding, but still allows the enzyme-substrate to bind and

the biochemical processes to continue. If more than one fungicide shares this target site, they might be cross-resistant to each other after the alteration.

The azole fungicides inhibit 14α-demethylase (CYP51), a P450 enzyme essential for the biosynthesis of ergosterol, a component of the cell membranes (Kwok & Loeffler, 1993). For this reason, they are named 14α-demethylation inhibiting fungicides (DMIs). Mutations in the encoding gene of this protein, *Mg*CYP51, leading to target site changes are the most common mechanism of azole resistance in *Z. tritici*, and they are often found in many different combinations. They can affect the efficiency of some azole compounds, but the cross-resistance is incomplete across the whole class of DMIs (Cools *et al.*, 2013).

A recent review describes the more than 30 different mutations in *Mg*CYP51 and their contribution towards azole field resistance (Cools & Fraaije, 2013). The point mutation L50S causes a slight increase in the binding pocket volume of the enzyme, although it is not associated with azole resistance (Mullins *et al.*, 2011), and also does not affect the enzyme activity (Cools *et al.*, 2010). The earliest reported alteration causing azole resistance is the amino acid substitution Y137F (replacement of tyrosine by phenylalanine at codon 137 of *Mg*CYP51), an alteration that prevents the binding of triadimenol (Leroux *et al.*, 2007). This residue alteration is nowadays rare in Western Europe, due to the replacement of triadimenol by newer azole compounds exerting a different pressure on the evolution of *Mg*CYP51 (Stammler *et al.*, 2008).

Some changes between Y459 to Y461 residues are very common and frequently associated with other substitutions in resistant strains. They are a precondition to the accumulation of alterations that alone would be lethal, such as I381V and V136A, but the enzyme activity is restored when associated with alterations in the Y459-Y461 area of the protein (Cools *et al.*, 2010). Cools et al. (2011) reported a reduction in sensitivity to all azoles, except prothioconazole, in *Z. tritici* isolates carrying *Mg*CYP51 variants with alterations L50S and Y461S. Additionally, Leroux et al. (2007) found strains carrying the double deletion of two *Mg*CYP51 residues, ΔY459/G460, as being resistant towards DMIs.

Strains of *Z. tritici* carrying the V136A substitution are less sensitive to prochloraz (Leroux *et al.*, 2007), but still sensitive to tebuconazole. In contrast, I381V confers a lower sensitivity to most triazoles, including tebuconazole, but enhances the sensitivity to prochloraz (Fraaije *et al.*, 2007). The emergence and accumulation of I381V in *Z. tritici* field populations coincided with a shift in resistance to tebuconazole (Lucas *et al.*, 2015). Unfortunately, isolates carrying both mutations soon evolved, and both mutations are now also often found in combination with D134G and/or S524T which increases the insensitive to both epoxiconazole and prothioconazole (Cools & Fraaije, 2013). The alteration D107V can also accommodate the lethal mutation I381V in the absence of any Y469-Y461 change (Stammler et al. 2008; Cools & Fraaije 2013). The combination of different *Mg*CYP51 mutations is a selection process driven by the treatment with different azoles and azole mixtures over time. It has resulted in the prevalence of more complex genotypes in European populations, like variant [L50S, S188N, A379G, I381V, ΔY459/G460 & N513K] (Stammler *et al.*, 2008; Huf *et al.*, 2018).

Benzimidazole (MBC) resistance is also correlated to a point mutation in the target site encoding gene, *β-tubulin* (Ma & Michailides, 2005). The β-tubulin alteration associated with *Z. tritici* MBC field resistance is E198A*,* first detected in 1984 and linked with poor STB control (Griffin & Fisher 1985). It is a mutation with no apparent fitness costs, still found at high frequencies, even in the absence of MBC use (Lucas & Fraaije, 2008).

The Quinone outside inhibitor (QoIs) fungicides bind to the cytochrome *bc1* enzyme complex in the inner mitochondrial membrane of fungi, at the Qo site, inhibiting mitochondrial respiration (Bartlett *et al.*, 2002). QoI resistance is conferred by point mutations in the cytochrome b (*cyt b*) gene, resulting in F129L, G137R or G143A, without compromising enzyme activity (Ma & Michailides 2005; FRAC 2006). Because all the QoIs bind similarly to the Qo site they are cross-resistant (Bartlett *et al.*, 2002). The single mutation G143A causes a high level of resistance, being the only one linked with STB field control failures, whereas F129L and G137R are associated with lower resistance factors (Brent & Hollomon, 2007). The first report of *Z. tritici* insensitivity to QoIs in the UK was in 2002, but retrospective DNA

testing of stored infected leaf samples showed that G143A could already be detected in 2001 (Lucas & Fraaije, 2007). Using spore trapping combined with DNA diagnostics, the selection and spread of QoI resistant alleles through airborne ascospores was demonstrated in a field trial (Fraaije *et al.*, 2003). The G143A allele is now widespread and QoIs fungicides are no longer efficient against STB in NW-Europe (Lucas *et al.*, 2015).

The succinate dehydrogenase inhibitors (SDHIs) were first discovered more than 40 years ago. More recently a second generation of these compounds has been released, with an increased spectrum of control. Succinate dehydrogenase is the complex II of the mitochondrial respiration chain and consisted of four subunits (A, B, C and D), of which B, C and D form the SDHIs binding site (Keon *et al.*, 1991). Single amino acid changes in SdhB, SdhC or SdhD of *Z. tritici* were found in less sensitive lab mutants. Two of these alterations, B-H267L and C-N86K showed cross-resistance to all four SDHIs tested (Fraaije *et al.*, 2012). Some *Z. tritici* field isolates with *Sdh* mutations conferring reduced sensitivity to the SDHI fungicides have already been detected in Europe, such as B-N225T, B-T268I, C-T79N, C-W80S, C-N86S and C-H152R (FRAG-UK, 2015; FRAC, 2016, 2019; Dooley *et al.*, 2016a; Kirikyali *et al.*, 2017; Rehfus *et al.*, 2018).

1.3.2 Overexpression of the target protein

Target-site overexpressing seems to be a common fungicide resistance mechanism to azoles in clinical and plant pathogenic fungi. Overexpression of CYP51 has already been reported in many different fungi, and are caused by alterations in the regulatory region of the target protein-encoding gene (Ma & Michailides, 2005). CYP51 overexpression in *Z. tritici* has been suggested (Stergiopoulos *et al.*, 2003) as a mechanism to azole resistance, and different inserts in its promoter have been identified (Leroux & Walker, 2011). However, a clear azole insensitivity phenotype linked to CYP51 overexpression has only been demonstrated for a 120 bp CYP51 promoter insert (Cools *et al.*, 2012). As a fungicide resistance mechanism, CYP51 overexpression will affect the efficacy of all azoles. Nevertheless, because the 120 bp CYP51 promoter insert has only been found in one particular CYP51 variant, [L50S,

S188N, I381V, ΔY459/G460 & N513K], which is not highly insensitive to most azoles, higher doses of the most effective azoles will still control these CYP51 overexpressing strains (Cools *et al.*, 2012, 2013).

1.3.3 Efflux pump overexpression

There are two main types of membrane transporter proteins associated with reduced accumulation of fungicides: ATP-binding cassette (ABC) transporters, that hydrolyse ATP to export compounds, and major facilitator superfamily (MFS) transporters, that use the proton gradient across the membrane to translocate substrates (Leroux & Walker, 2011). The involvement of these integral membrane proteins in drug efflux outside the cell affects fungicides with different modes of action, and so is known as multiple drug resistance (MDR), a well-known mechanism in clinical fungi and human cancer cells (Morschhäuser, 2010). In *Z. tritici* the function of one MFS transporter (*MgMFS1*) and five ABC transporters (*Mg*Atr1-5) were characterized using gene knock-out studies of lab mutants (de Waard *et al.*, 2006; Roohparvar *et al.*, 2007, 2008). *Z. tritici* ABC-transporters can also provide protection against chemical toxic compounds (Zwiers *et al.*, 2003). The importance of *MgMFS1* was recently demonstrated for field strains where azole and SDHI insensitivity was linked to *MgMFS1* overexpression due to either three different types of insert (Type I: 519 bp; Type II: 339 or 369 bp; and Type III: 149 bp) in the promoter sequence (Omrane *et al.*, 2015, 2017).

1.4 Project aims

Wheat yield losses due to Septoria tritici blotch can be reduced by adopting an integrated management approach based on good cultivation practices, host resistance, and chemical control. However, multiple fungicide classes have shown a reduction in field efficacy in some regions. The status of fungicide resistance in South American populations of *Zymoseptoria tritici* is not known. Hence, further investigating the evolution of fungicide resistance is important to appropriate disease management. Thus, the overall aim of this thesis was to investigate the current status and further potential of evolution of resistance to fungicides in *Z. tritici* field populations from South America. To address this goal the following specific objectives were accomplished:

Chapter 2: Test *in vitro* sensitivity of South American field isolates to different fungicide classes (MBC, QoI, azoles, SDHIs and multisite) used to control Septoria tritici blotch and compare them against populations from other continents.

Chapter 3: To elucidate molecular mechanisms underlying fungicide resistance and clarify the evolutionary history of azole-resistant *CYP51* alleles.

Chapter 4: To characterize *Z. tritici* population diversity and structure with neutral molecular markers to explain the emergence of azole resistance in South America.

Chapter 2: Fungicide sensitivity of *Zymoseptoria tritici* field populations from South America

2.1 Introduction

In South America, like in the rest of the world, huge increments in wheat yield were obtained after the green revolution with the introduction of dwarfing genes on wheat breeding programs. Possibly because of shorter varieties and increased nitrogen usage, Septoria tritici blotch also gained importance in wheat-growing regions (Kraan & Nisi, 1993). With increased yield, it is expected an increase in the mass of stubble left in the field after harvest. *Z. tritici* can survive for extended periods on wheat straw in the form of perithecia (Sanderson, 1972). It has been observed in Argentina that a wheat/soybean sequence under no-tillage or a wheat following wheat system allows inoculum of several necrotrophic fungi, including *Z. tritici*, to survive until the next wheat season (Rosa *et al.*, 2013). The infected stubble left over the soil will discharge ascospores to be dispersed with the wind and so spread the inoculum over a large area (Shaw & Royle, 1989). Although the factors leading to an increase in STB are not completely understood, with the advance of no-tillage systems higher severity of STB in wheat fields of South America is expected. Consequently, maintaining the effectiveness of fungicides to control this disease is important to wheat production in South America.

In Argentina, wheat is the most important winter cereal and has been cultivated for over 100 years. The wheat-growing region is situated in a region between 31 and 40^{o} of latitude south, with typical dry winter and increased rainfall from October coinciding with the reproductive stage of the crop. Most cultivars are spring wheat. Historically STB was the most important wheat foliar disease due to its presence and frequency in most areas, followed by *Puccinia graminis*, *Puccinia recondita*, *Fusarium spp*. and more sporadically *Puccinia striiformis* (Kraan & Nisi, 1993). As a result of a large and extensive wheat-growing region (approximately 5 million hectares) with climate conditions favourable to the development of STB, and mostly moderately susceptible cultivars, fungicides have been used to control foliar diseases since before 1984 with a major increase from 1996 onwards (Galich *et al.*, 1990; Rosa *et al.*, 2013). According to Galich *et al.* (1990), efficient chemical control of STB in the period 1984-86 was

obtained with a single spray of one of the azoles propiconazole or triadimenol, either alone or mixed with mancozeb. In the 1990s a series of new azoles were introduced into the market and later strobilurins. These became the main tools to control foliar wheat diseases in Argentina (Rosa *et al.*, 2013) so that around 2000 50% of the commercial products were azoles and 50% a formulated mix of azoles and strobilurins (Campos, 2008). At present, mixtures of azoles and strobilurins still represent most of the fungicide usage on wheat, alongside new formulations of these with the addition of SDHIs (INTA - Argentina, 2018).

The systemic fungicides were introduced into Uruguay with the MBC benomyl in 1968. From 1983 to 1985 azoles propiconazole, triadimenol, fenetrazole, and triadimefon were introduced and tested to control STB (Germán *et al.*, 1990). Later, in 1991, the new azoles tebuconazole, epoxiconazole and prochloraz were introduced for STB control, and from 1998 strobilurins become commercially available, either alone or mixed with azoles (Ackermann, 1996, 2010). As in Argentina, in Uruguay wheat foliar diseases have been controlled with mixtures of azoles and strobilurins during the last decade, and currently, the most promising field control efficiency is being achieved with mixtures of these with the new SDHIs (INIA - Uruguay, 2017).

STB is a common disease in all wheat-growing regions in Chile, but the southernmost areas of the country present more suitable climate conditions for *Z. tritici* and therefore higher disease pressure (Caglevic, 1982; Andrade, 1995). Fungicides have been tested for control of STB in Chile since 1978, when mancozeb, carbendazim, benomyl, and triadimefon were experimented with. Between 1978 and 1980 Gilchrist & Madariaga (1980) found good field control of STB with carbendazim, mixed with maneb, and triadimefon. In 1985 other azole compounds were introduced, including propiconazole and prochloraz, with tebuconazole introduced in 1990 (Andrade, 1990, 1995; Mellado, 1990). Andrade (1990) found the best field efficiency with propiconazole, flutriafol and prochloraz sprayed at flag leaf (GS39) with the maximum of one additional spray per season. In the last decade, chemical control of

wheat diseases in Chile has relied on azoles, strobilurins, and more recently SDHIs, generally in a formulated mixture of molecules from at least two different classes (Jobet *et al.*, 2013).

Control of STB is heavily dependent on fungicides in all wheat growing countries where this disease has some impact on wheat yield. Unfortunately, azole, MBC and QoI resistance have developed and become widespread throughout many countries in Europe (Leroux *et al.*, 2007; Torriani *et al.*, 2009; Drabešová *et al.*, 2013; Cools & Fraaije, 2013; Lucas *et al.*, 2015) and also in other continents, like North America (Estep *et al.*, 2013, 2015; Hagerty *et al.*, 2017) and Oceania (Stewart *et al.*, 2014; McDonald *et al.*, 2019). Poor field control of STB with MBCs (Ackermann, 2010) and the presence of *Z. tritici* strains with reduced sensitivity to tebuconazole (Cordo *et al.*, 2017) have been reported in South America. Due to the long history of fungicide use to control STB in South America, it is expected that *Z. tritici* field populations also have evolved resistance against some of these fungicide classes.

The aim of the work reported in this chapter was to test the hypothesis that South American *Z. tritici* populations have evolved resistance to different fungicide classes used to control STB over the years. To test this hypothesis, field populations of *Z. tritici* were sampled from four locations in three South American countries representing two regions more or less isolated from each other. The fungicide sensitivity spectrum in these samples was determined and compared with populations sampled in other continents.

2.2 Material and Methods

2.2.1 Sampling locations

To achieve the aims proposed in this thesis *Z. tritici* field populations were sampled in three South American countries where STB causes a high impact in wheat yield and fungicides have been extensively used to control the disease over the years. All wheat leaves came from naturally infected commercial fields sampled in 2016 before fungicide applications. Samples were composed of 50 to 100 diseased leaves from a single field, sampled uniformly across the field from plants separated at least three meters apart from each other to avoid clonality. From Argentina, the sample was taken from a

commercial field near to Gualeguaychú city, located in the eastern region of Entre-Rios province. In Uruguay, two samples were collected in different commercial fields located six km apart in the Colonia Department. The sample from Chile was collected in a commercial field located near to Perquenco town in southern Chile's Araucanía Region (Figure 2.1). The South American wheat fields were located in regions of Humid Subtropical climate - Cfa (Uruguay and Argentina) and Oceanic climate - Cfb (Chile) (Kottek *et al.*, 2006), thus favourable for STB epidemics.

To compare South American populations of *Z. tritici* with other global populations, infected wheat leaves were also sampled across Europe, North America, and New Zealand. To represent NW-European populations of *Z. tritici* that have been long selected for fungicide resistance, one population was sampled at the Rothamsted Farm, located in Harpenden, England. Two other winter wheat fields were sampled in Eastern European countries where fungicide usage is less intense than Western Europe: From the Czech Republic, infected wheat leaves were sampled in Kroměříž, while in Slovakia the leaves were sampled in a commercial field in Piešťany. In North America, *Z. tritici* populations from Oregon-USA have been extensively studied regarding fungicide resistance. Therefore, one wheat winter field sampled close to the town of Corvallis, in the Willamette Valley of Oregon, was included in this study. To compare it with a population in which the status of fungicide resistance is unknown, another sample was collected in the USA in 2018, in a winter wheat field located at East Lansing, Michigan. Fungicide resistance has also been reported in New Zealand populations of *Z. tritici*, thus one sample was taken in 2017 from a commercial wheat field in Aorangi, located at the South of the North Island in New Zeeland (Figure 2.1).

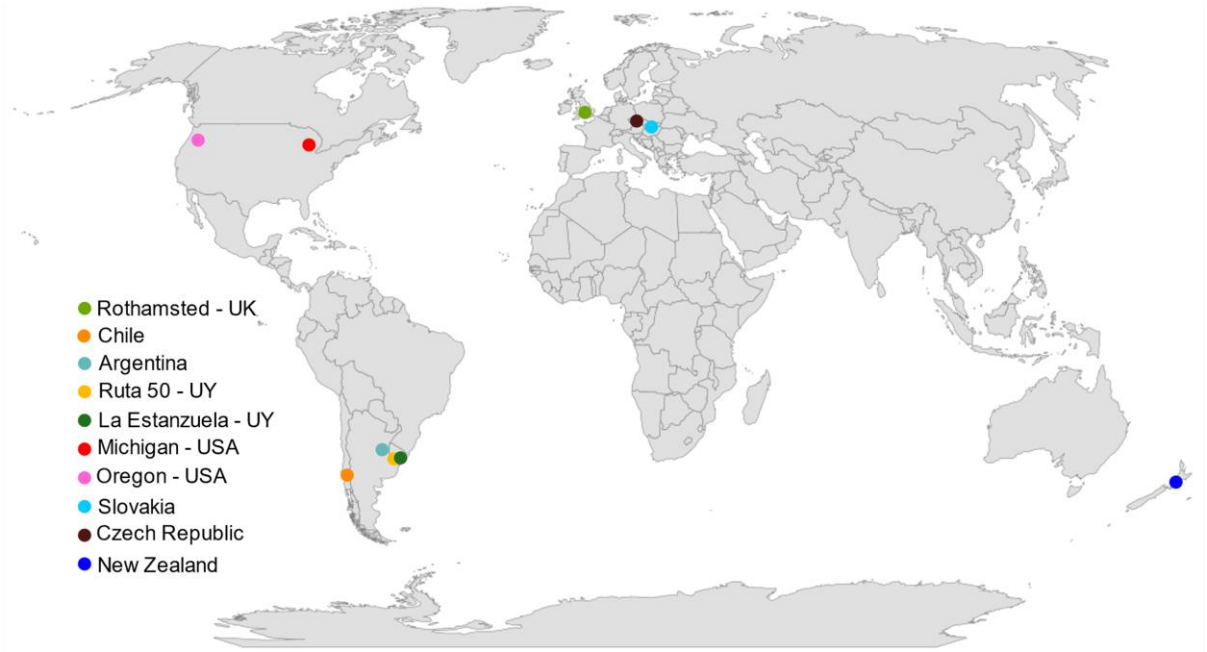

Figure 2.1 Sampling locations.

2.2.2 Fungal Isolation

In the laboratory, a lesion of STB was cut from each leaf, attached to filter paper, and incubated in a petri dish for 24 h, in the dark, at room temperature, and under high humidity conditions, to induce the release of spores from pycnidia. A cirrhus was collected with tweezers from a single pycnidium and put in 30 µL of sterile distilled water to disperse the pycnidiospores. This spore suspension was plated out on Yeast extract Peptone Dextrose (YPD) agar medium (ForMedium™, Norwich, UK) amended with antibiotics penicillin G sodium and streptomycin sulfate (Sigma-Aldrich, Gillingham, UK) at a concentration of 100 µg/mL. After 14 days, a single colony was sub-cultured to new a YPD plate. Spores were harvested after seven days of incubation at 15°C in the dark and stored in 80% glycerol at -80°C. One strain was isolated per leaf sampled in the field. The isolates collection generating the populations from each sample of infected wheat leaves is described in Table 2.1.

Table 2.1 Populations of *Zymoseptoria tritici*.

Population Name	Number of isolates	Country	Location	Sample code	Year	Cultivar
Rothamsted	50	England	Harpenden	RR16	2016	Reflection
Chile	71	Chile	Araucanía	CHI	2016	Crac-baer
Argentina	67	Argentina	Entre Rios	ER	2016	DM Fuste
Ruta 50	53	Uruguay	Colonia	R50	2016	INIA 2375
La Estanzuela	42	Uruguay	Colonia	LE	2016	INIA LE 2438
Michigan	46	USA	East Lansing	MIC	2018	Ambassador
Oregon	57	USA	Corvallis	ORE	2016	Kaseberg
Slovakia	39	Slovakia	Piešťany	SLV	2016	Stelarka
Czech Republic	47	Czech Republic	Kroměříž	CR	2016	Turandot
New Zealand	51	New Zealand	Aorangi	AOR	2017	Katch 42 (KWW42)

2.2.3 Fungicide sensitivity testing

Sensitivity tests were carried out with fungicides from the following classes: DMIs (epoxiconazole, tebuconazole, and prochloraz), QoI (azoxystrobin), SDHIs (bixafen and fluopyram) and MBC (carbendazim). Also, one multisite fungicide (chlorothalonil) and one efflux transporter substrate (tolnaftate) were tested. Technical grade fungicides were purchased from Sigma-Aldrich and dissolved in dimethylsulphoxide (DMSO). For all compounds, dilution series were made and 12 concentrations, including untreated, tested in Sabouraud liquid media (Sabouraud Dextrose Broth – Oxoid, Basingstoke, UK) (Table 2.2). The concentration range of each fungicide was chosen in such a way that it is was possible to determine the EC_{50} values of most, if not all, isolates, from the most sensitive to the more resistant. The fungicides tolnaftate and carbendazim were tested at a single concentration, 10 and 1 µg/mL respectively. These discriminatory doses were chosen based on results from pilot experiments described in section 2.3.1.8.

Z. tritici spores stored at -80°C in 80% glycerol were plated out on YPD agar and kept in the dark at 15°C. After 7 days, these spores were harvested with a 5 µL loop and suspended in sterile distilled water. The concentration of spores was determined using a haemocytometer (Webber Scientific International, Middlesex, UK) and adjusted to a concentration of $2.5x10^6$ spores/mL. Aliquots of 100 µL of increasing doses of fungicide-amended Sabouraud liquid media were pipetted in flat bottomed sterile 96-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany). 100 µL of spore suspension was added to the cells and incubated in the dark at 21°C. After four days, fungal growth was measured using absorbance readings at 630 nm with a FLUOstar OPTIMA microplate reader (BMG Labtech GmbH, Offenberg, Germany). Absorbance was measured in a well-scanning mode with a 2×2 matrix of scanning points set at 3 mm diameter. The dose-response curve was fitted with the OPTIMA software v2.200R2 and the concentration that inhibits 50% of the fungal growth (EC_{50} in µg/mL) was calculated. For carbendazim and tolnaftate, the fungicides are pipetted in one column of the microtiter plate, in a single concentration, and the next column is pipetted only with the spore suspension and unamended liquid media, as a control. The plate reader measures the fungal growth in both columns, which is compared to score the strains as sensitive or not based on the fungal growth in the presence of fungicide. The isolates IPO323, IRE30 and NT321.17, with known fungicide sensitivity, were used as references. The presented fungicide EC_{50} values are the averages of two replicates for each isolate.

Table 2.2 Fungicides dilution series in µg/mL.

Fungicide	Microtiter plate columns											
	1	2	3	4	5	6	7	8	9	10	11	12
Tebuconazole	0	3.0E-3	8.0E-3	2.0E-2	6.0E-2	1.7E-1	4.7E-1	1.3	3.6	9.9	27.3	75
Epoxiconazole /Azoxystrobin	0	1.4E-4	5.0E-4	2.0E-3	7.0E-3	3.0E-2	1.0E-1	3.8E-1	1.4	5.3	20	75
Prochloraz	0	1.5E-6	7.7E-6	3.8E-5	1.9E-4	9.6E-4	4.8E-3	2.4E-2	0.1	0.6	3	15
Chlorothalonil	0	1.0E-2	2.0E-2	4.0E-2	8.0E-2	1.6E-1	0.3	0.6	1.2	2.5	5	10
Bixafen	0	2.3E-4	9.0E-4	3.6E-3	1.5E-2	5.8E-2	2.4E-1	9.4E-1	1.9	3.7	7.5	15
Fluopyram	0	1.7E-3	5.0E-3	1.5E-2	4.5E-2	1.4-E1	0.4	1.2	3.7	11	33.3	100

2.2.4 Data analysis

The cumulative frequency distributions of calculated fungicide EC_{50} values for South American populations were analysed with the Anderson-Darling test (AD-test) using Ksamples package v1.2.9 in R environment (Fritz & Scholz, 2019). The AD-test detects differences in the spread of cumulative frequency distributions among populations. Thus, it was used to test whether the fungicide cumulative frequency distributions from the four South American populations were part of a single population or not. Further pairwise comparisons were made between populations with Kolmogorov-Smirnov two-sample test (KS-test) in GenStat v19.1.2 (VSN International Ltd. United Kingdom). The KS-test looks for the greatest difference between the two cumulative distributions. Cross-resistance between EC_{50} values of pairs of fungicides were tested with Spearman's correlation coefficient in SigmaPlot v14.0.

To compare the fungicide sensitivity of South American populations in relation to other regions of the world the samples were grouped according to their continental origin as South America, North America, Eastern Europe, New Zealand, and Western Europe. The mean fungicide sensitivity for groups and populations of *Z. tritici* was compared using ANOVA in GenStat v19.1.2. For fungicides azoxystrobin and carbendazim, the strains were categorized as sensitive or resistant, thus a Generalized Linear Model (GLM) was applied to compare the proportions of sensitive and resistant isolates. The GenStat

software v19.1.2. was used to construct a generalized linear model with a Bernoulli distribution and a logit link to estimate the change in the proportion of resistant *Z. tritici* isolates between groups and populations.

2.3 Results

2.3.1 Fungicide sensitivity in South American *Zymoseptoria tritici* field populations

2.3.1.1 Sensitivity profiles of azole fungicides

Azole sensitivity (EC_{50} values in liquid medium) was determined for all *Z. tritici* field isolates sampled for each of the South American populations described previously, in section 2.2.3. In general, all these populations had mean log EC_{50} values greater than the reference isolate IPO323 for the three azoles tested (Table 2.3).

The baseline of log fungicide sensitivity levels for unselected pathogen populations commonly follows a Gaussian distribution (Russell, 2004). Frequency histograms for Chile, Argentina and Ruta 50 were negatively skewed, with more isolates with epoxiconazole EC_{50} values in the upper half of the range, whereas the population from La Estanzuela had a distinctly bimodal distribution of sensitivity to epoxiconazole (Figure 2.2). Although, for tebuconazole (Figure 2.3) and prochloraz (Figure 2.4), Chile had a bimodal distribution of the log EC_{50} values, in contrast, Argentina, Ruta 50 and La Estanzuela isolates showed frequency distributions of log EC_{50} values in a unimodal shape skewed to lower EC_{50} values. Within the population from Chile, 10, 29 and 7% of the isolates had greater EC_{50} values than any other isolate found in the South American populations for epoxiconazole, tebuconazole, and prochloraz, respectively.

Isolates from Chile had a wider spectrum of azole sensitivity and higher resistance factors compared to the other populations from South America. The less sensitive strains from Chile were 1200, 666 and 228-fold higher than IPO323 sensitivity for epoxiconazole, tebuconazole, and prochloraz, respectively (Table 2.4).

According to the K-sample Anderson-Darling test the cumulative frequency distributions were significantly different between populations for epoxiconazole (Anderson-Darling K-sample test, AD = 12.08, T.AD = 6.97, $p < 0.001$), tebuconazole (Anderson-Darling K-sample test, AD = 18.06, T.AD = 11.56, $p < 0.001$) and prochloraz (Anderson-Darling K-sample test, AD = 25.33, T.AD = 17.14, $p < 0.001$).

The pairwise comparisons showed that the epoxiconazole cumulative frequency distribution from Chile is different from Argentina (Figure 2.5; Kolmogorov-Smirnov two sample test, D=0.31, χ^2 approximation = 13.0, 2 df, p = 0.001), Ruta 50 (Figure 2.5; Kolmogorov-Smirnov two sample test, D=0.27, χ^2 approximation = 8.7, 2 df, p = 0.01) and La Estanzuela (Figure 2.5; Kolmogorov-Smirnov two sample test, D=0.29, χ^2 approximation = 9.1, 2 df, p = 0.01). Interestingly, the two Uruguayan populations that were sampled only six km apart, Ruta 50 and La Estanzuela, had different cumulative curves for epoxiconazole sensitivity (Figure 2.5; Kolmogorov-Smirnov two sample test, D=0.32, χ^2 approximation = 9.8, 2 df, p = 0.008).

Due to its bimodal shape, the tebuconazole cumulative frequency distribution of Chilean isolates was significantly different from Argentina (Figure 2.6; Kolmogorov-Smirnov two sample test, D=0.41, χ^2 approximation = 23.5, 2 df, $p < 0.001$), Ruta 50 (Figure 2.6; Kolmogorov-Smirnov two sample test, D=0.46, χ^2 approximation = 26.2, 2 df, $p < 0.001$) and La Estanzuela (Figure 2.6; Kolmogorov-Smirnov two sample test, D=0.37, $\chi2$ approximation = 14.2, 2 df, $p < 0.001$). As seen for epoxiconazole, the Uruguayan populations from La Estanzuela and Ruta 50 had different distributions of sensitivity for tebuconazole (Figure 2.6; Kolmogorov-Smirnov two sample test, D=0.37, $\chi2$ approximation = 12.9, 2 df, p = 0.002).

Corresponding to the other azoles, pairwise comparisons showed that the frequency of isolates with greater prochloraz EC_{50} values was higher in Ruta 50 than in La Estanzuela (Figure 2.7; Kolmogorov-Smirnov two sample test, D=0.34, $\chi2$ approximation = 10.6, 2 df, p = 0.005). Isolates sampled from Chile were again differently distributed to prochloraz sensitivity than isolates from Argentina (Figure 2.7; Kolmogorov-Smirnov two sample test, D=0.30, $\chi2$ approximation = 12.2, 2 df, p = 0.002), Ruta 50 (Figure

2.7; Kolmogorov-Smirnov two sample test, D=0.50, $\chi 2$ approximation = 30.6, 2 df, $p < 0.001$) and La Estanzuela (Figure 2.7; Kolmogorov-Smirnov two sample test, D=0.30, $\chi 2$ approximation = 9.8, 2 df, $p = 0.007$).

Table 2.3 Azole sensitivities of South American *Zymoseptoria tritici* field populations from Chile, Argentina, Ruta 50-UY and La Estanzuela-UY.

Population	n	Epoxiconazole			Tebuconazole			Prochloraz		
		Log_{10} EC_{50}±SE[1] (mean)	SD[2]	EC_{50}[3] (μg/mL)	Log_{10} EC_{50}±SE[1] (mean)	SD[2]	EC_{50}[3] (μg/mL)	Log_{10} EC_{50}±SE[1] (mean)	SD[2]	EC_{50}[3] (μg/mL)
Chile	71	-1.449 ± 0.109	0.914	0.035	-0.507 ± 0.140	1.179	0.308	-2.058 ± 0.098	0.828	0.009
Argentina	67	-1.354 ± 0.086	0.701	0.044	0.120 ± 0.065	0.537	1.318	-2.051 ± 0.096	0.785	0.009
Ruta 50	53	-1.281 ± 0.101	0.733	0.052	0.264 ± 0.041	0.300	1.836	-1.728 ± 0.056	0.407	0.019
La Estanzuela	42	-1.727 ± 0.148	0.957	0.019	-0.105 ± 0.099	0.639	0.785	-2.081 ± 0.099	0.644	0.008
IPO323[4]	-	-3.350 ± 0.038	-	-	-1.925 ± 0.081	-	-	-3.247 ± 0.092	-	-

[1]EC_{50} are the mean of two replicates for each isolate.
[2]SD are in log scale.
[3]EC_{50} are the back transformation of Log_{10} EC_{50}.
[4]EC_{50} are the mean of 19 independent experiments.

Table 2.4 Range of azole resistance factors of South American *Zymoseptoria tritici* field populations from Chile, Argentina, Ruta 50-UY and La Estanzuela-UY.

Population	n	Epoxiconazole	Tebuconazole	Prochloraz
		(Range of RF)[1]	(Range of RF)[1]	(Range of RF)[1]
Chile	71	1.0 - 1200	0.33 - 666	0.14 - 228
Argentina	67	0.50 - 575	0.33 - 233	0.01 - 71.0
Ruta 50	53	1.25 - 475	16.6 - 315	0.28 - 114
La Estanzuela	42	0.75 - 750	0.46 - 248	0.06 - 57.0

[1]Resistance factor was calculated by dividing absolute values of the lowest EC_{50} and the highest EC_{50} in the range by IPO323 EC_{50}.

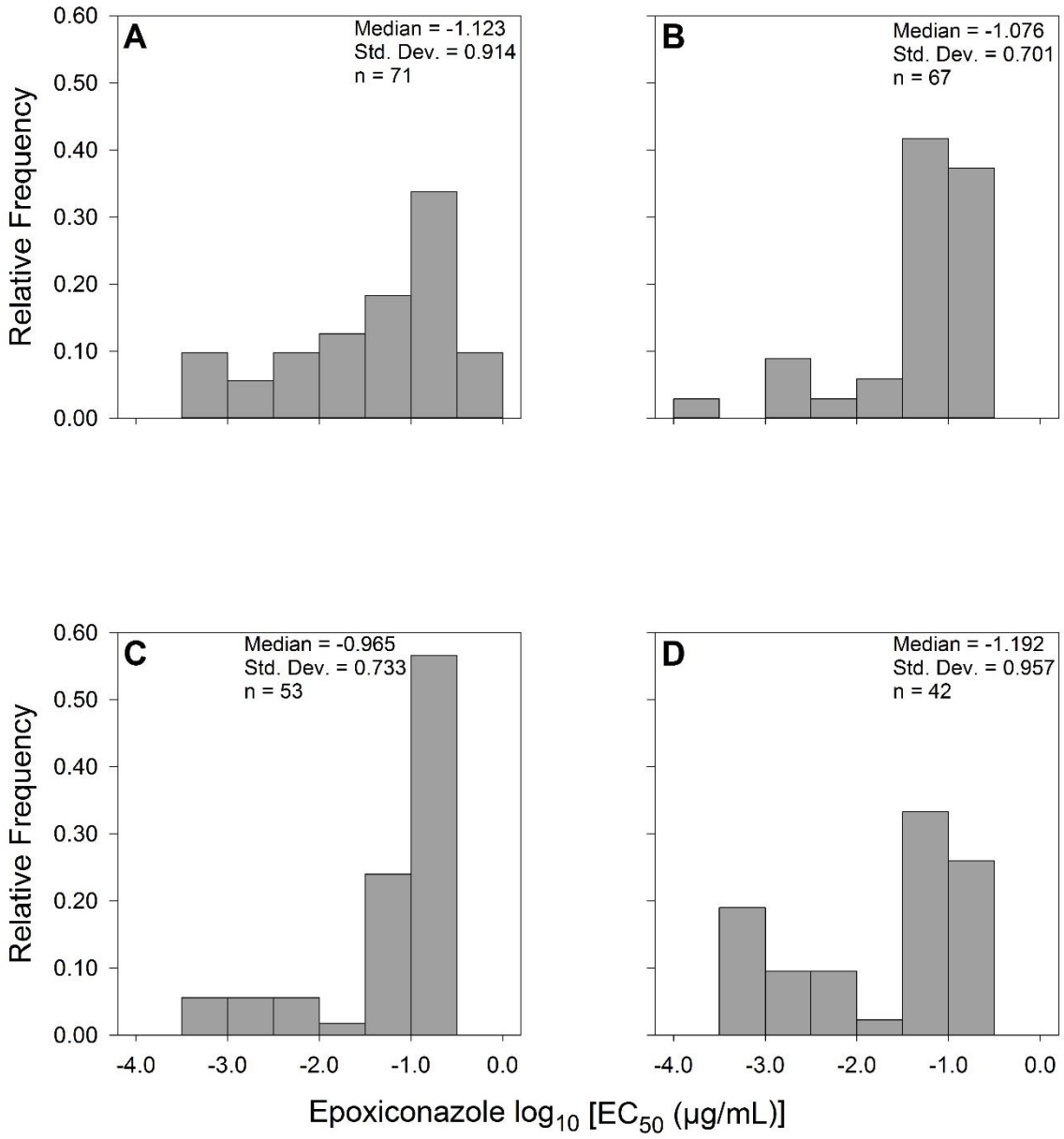

Figure 2.2 Frequency distribution of epoxiconazole log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A), Argentina (B), Ruta 50-Uruguay (C) and La Estanzuela-Uruguay (D).

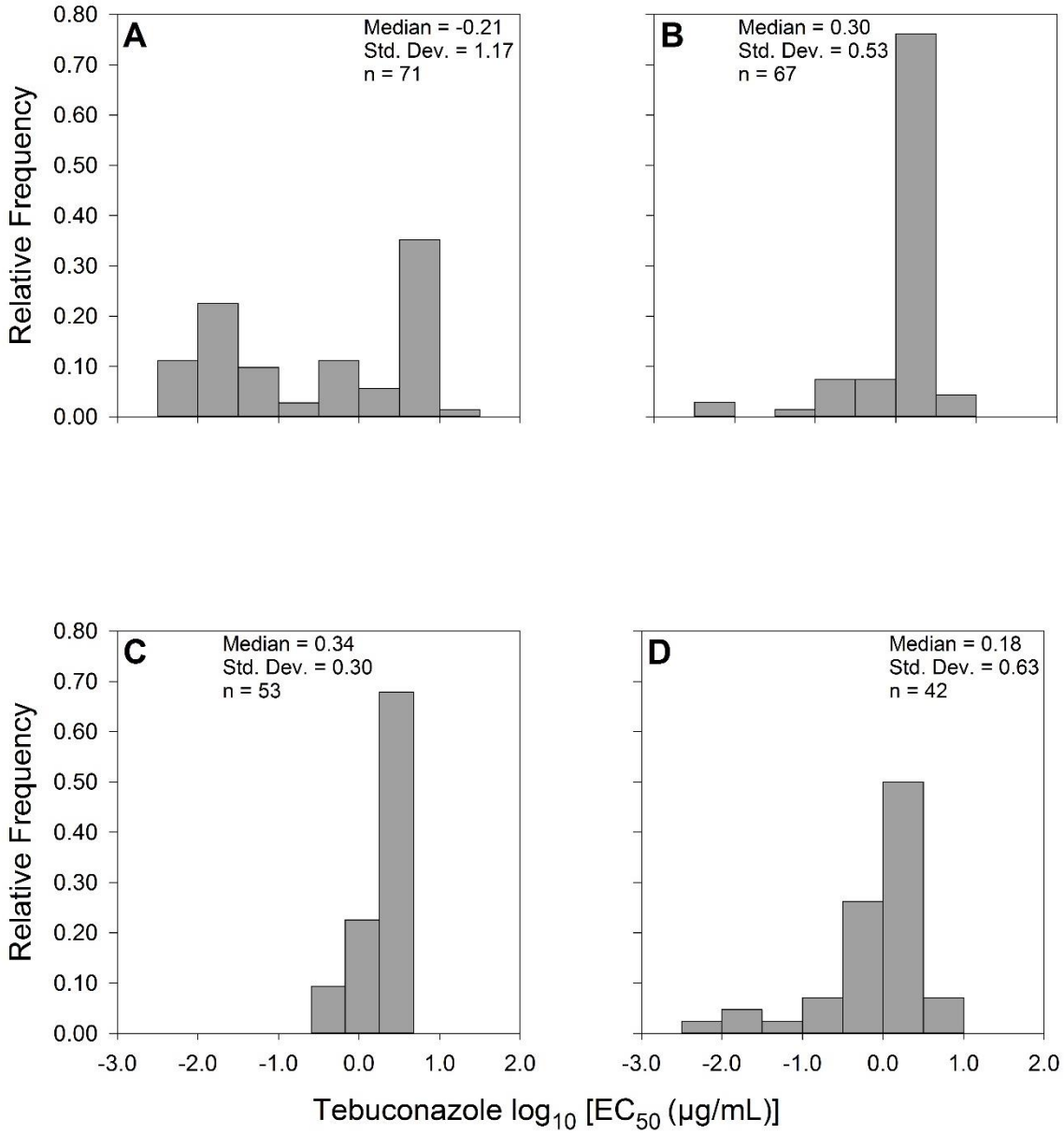

Figure 2.3 Frequency distribution of tebuconazole log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A), Argentina (B), Ruta 50-Uruguay (C) and La Estanzuela-Uruguay (D).

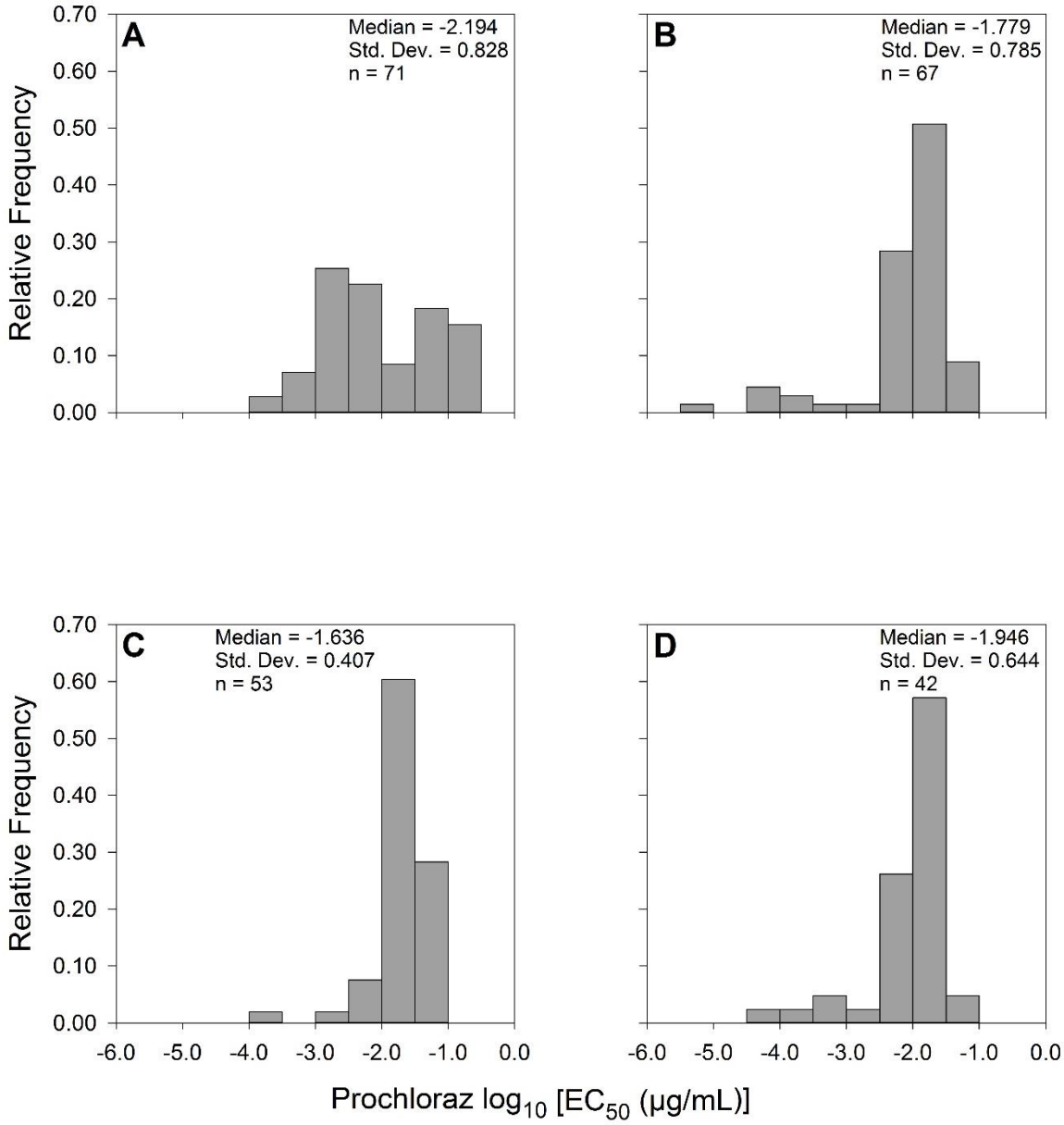

Figure 2.4 Frequency distribution of prochloraz log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A), Argentina (B), Ruta 50-Uruguay (C) and La Estanzuela-Uruguay (D).

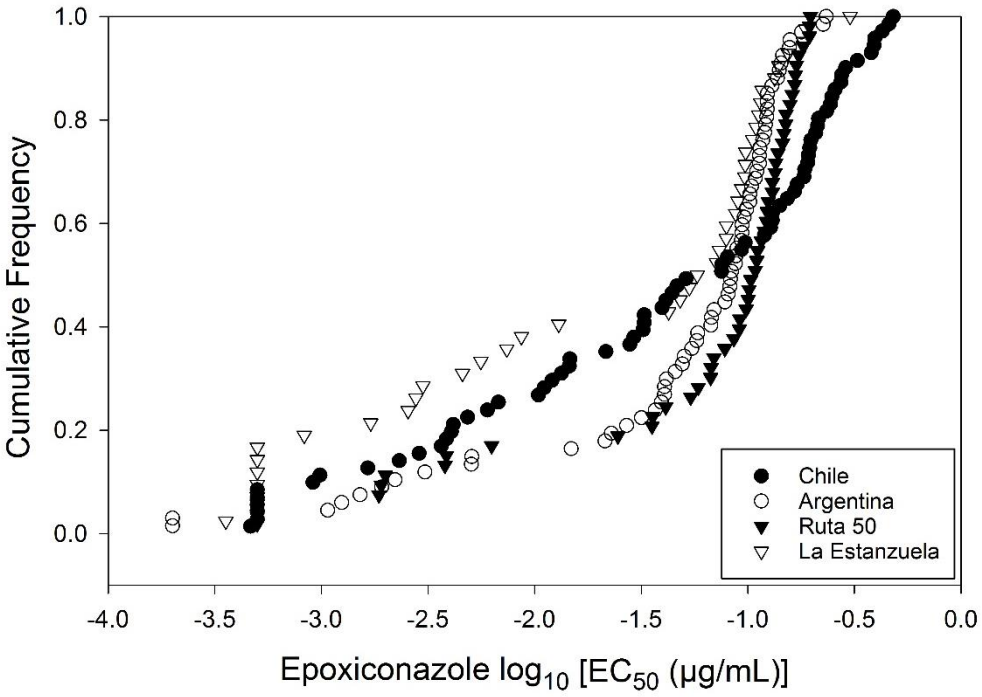

Figure 2.5 Cumulative frequency distribution of epoxiconazole log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53) and La Estanzuela-Uruguay (n=42).

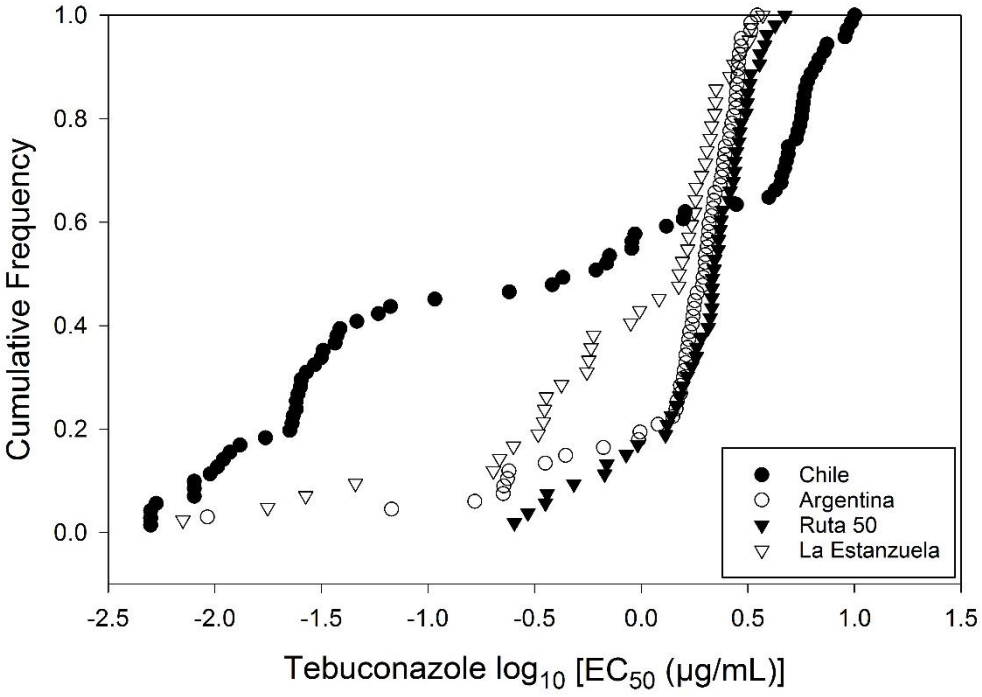

Figure 2.6 Cumulative frequency distribution of tebuconazole log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53) and La Estanzuela-Uruguay (n=42).

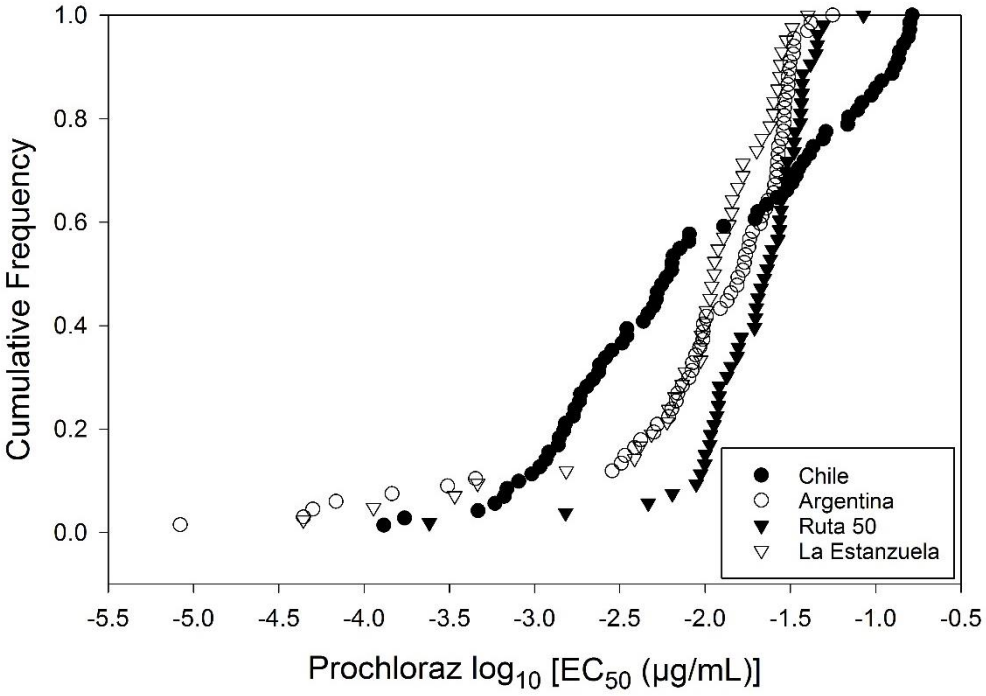

Figure 2.7 Cumulative frequency distribution of prochloraz log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53) and La Estanzuela-Uruguay (n=42).

2.3.1.2 Cross-resistance of azole fungicides

Epoxiconazole and tebuconazole EC_{50} values were positively and significantly correlated for Chile ($r = 0.80$, $p < 0.001$, $n = 71$), Argentina ($r = 0.71$, $p < 0.001$, $n = 67$), Ruta 50 ($r = 0.84$, $p < 0.001$, $n = 53$) and La Estanzuela ($r = 0.83$, $p < 0.001$, $n = 42$) (Figure 2.8). In the Chile population, some of the isolates were more sensitive for tebuconazole regardless of the epoxiconazole sensitivity, reflecting the bimodal distribution of sensitivity for tebuconazole in this population. A similar trend was observed in La Estanzuela, where many isolates were very sensitive for epoxiconazole and this was not correlated with tebuconazole sensitivity.

Epoxiconazole and prochloraz sensitivity were either uncorrelated, as for Chile ($r = -0.0005$, $p = 0.99$, $n = 71$) and La Estanzuela ($r = 0.23$, $p = 0.14$, $n = 42$), or weakly but significantly correlated, as for Argentina ($r = 0.48$, $p < 0.001$, $n = 67$) and Ruta 50 ($r = 0.45$, $p < 0.001$, $n = 53$) (Figure 2.9). A high proportion of Chile isolates had low sensitivity for epoxiconazole and this was not correlated with low sensitivity for

prochloraz. Meanwhile, part of the population from La Estanzuela had low sensitivity for prochloraz regardless of epoxiconazole EC_{50} values.

Sensitivity of tebuconazole and prochloraz was not correlated for isolates from Chile ($r = -0.22$, $p = 0.07$, $n = 71$) and weakly but significantly correlated for Argentina ($r = 0.48$, $p < 0.001$, $n = 67$), Ruta 50 ($r = 0.43$, $p = 0.001$, $n = 53$) and La Estanzuela ($r = 0.32$, $p = 0.04$, $n = 42$) populations (Figure 2.10). The bimodal distribution of EC_{50} values for tebuconazole and prochloraz in the population from Chile was now observed as three separated groups of isolates, the first one had higher EC_{50} values for prochloraz and low values for tebuconazole, the second one had high sensitivity for prochloraz and low sensitivity for tebuconazole, and between these a third group appeared to have positively correlated sensitivity levels for the pair of fungicides.

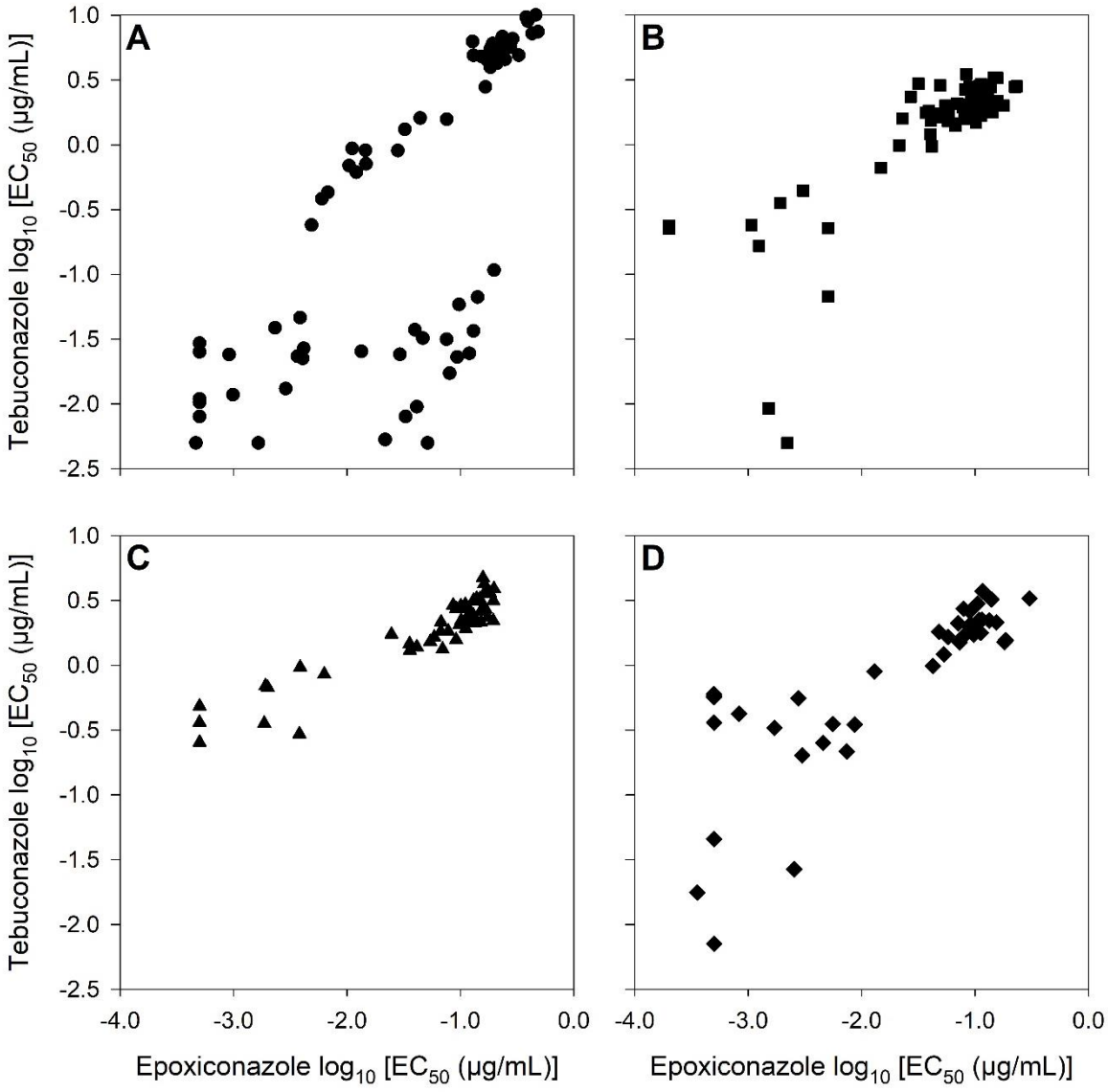

Figure 2.8 Correlation between epoxiconazole and tebuconazole log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A, n=71), Argentina (B, n=67), Ruta 50-Uruguay (C, n=53) and La Estanzuela-Uruguay (D, n=42).

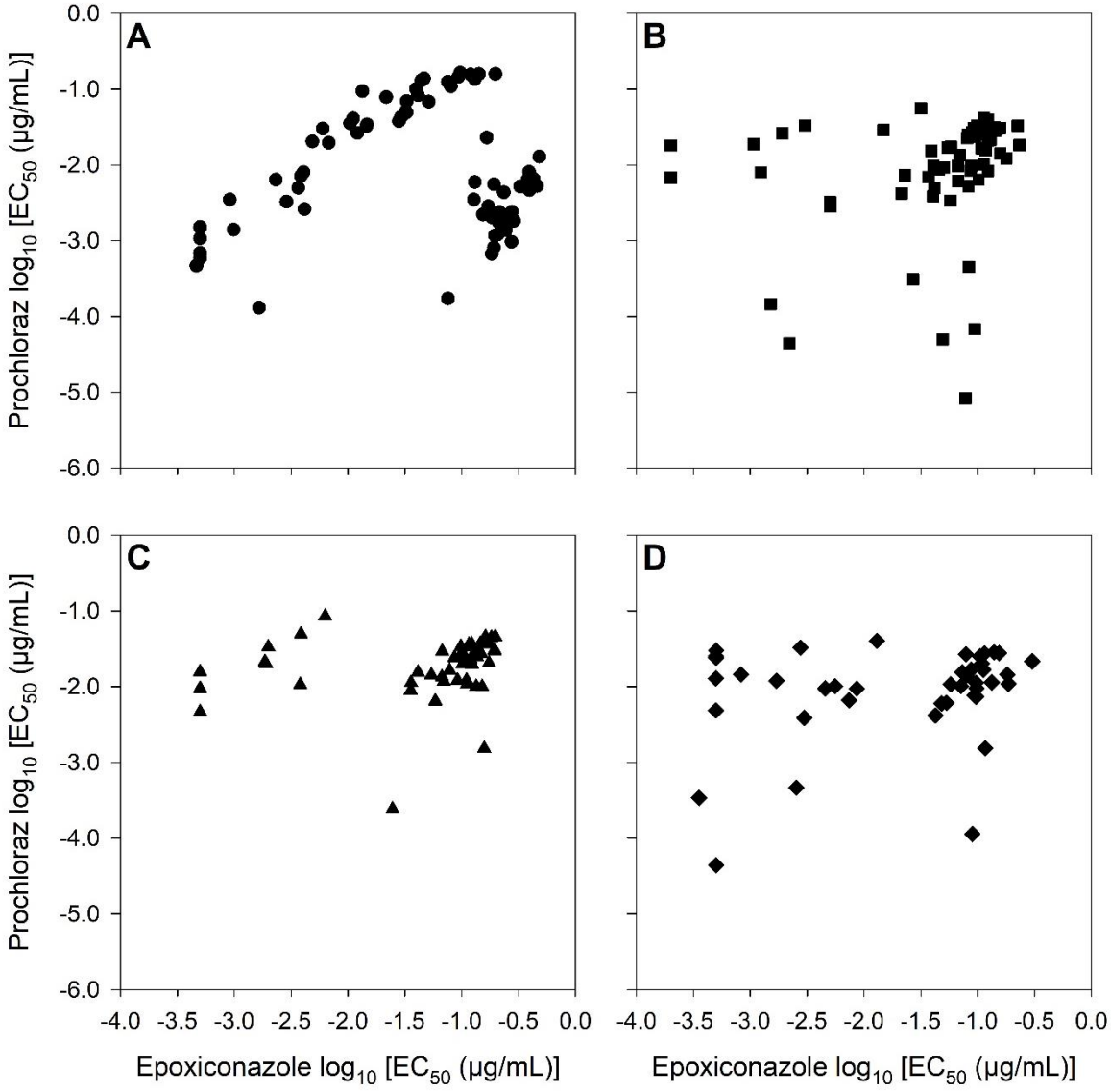

Figure 2.9 Correlation between epoxiconazole and prochloraz log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A, n=71), Argentina (B, n=67), Ruta 50-Uruguay (C, n=53) and La Estanzuela-Uruguay (D, n=42).

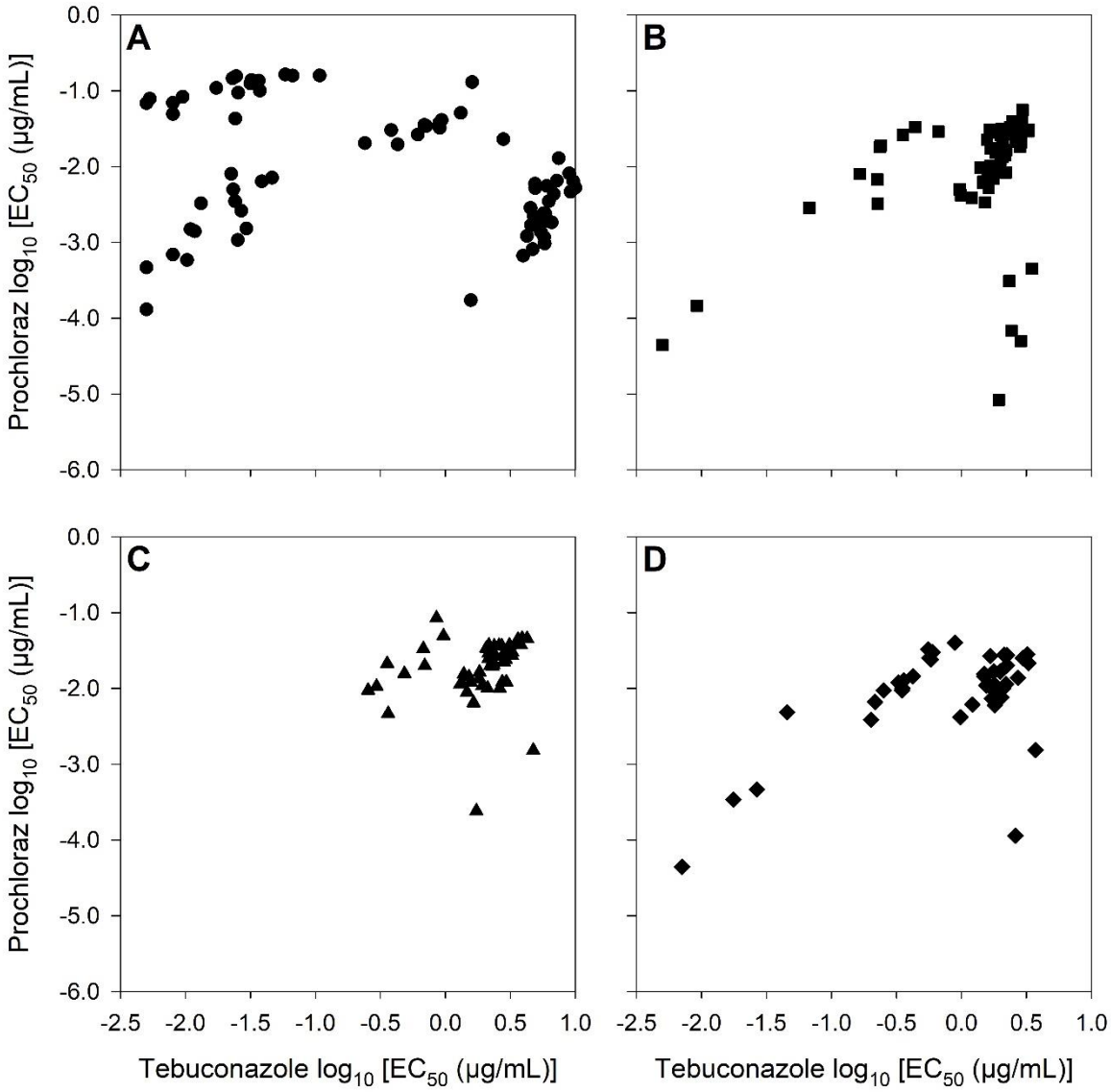

Figure 2.10 Correlation between tebuconazole and prochloraz log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A, n=71), Argentina (B, n=67), Ruta 50-Uruguay (C, n=53) and La Estanzuela-Uruguay (D, n=42).

2.3.1.3 Sensitivity profiles to azoxystrobin

The distribution of azoxystrobin log EC_{50} values for all four South American populations showed a distinctly bimodal shape (Figure 2.11). The relatively large differences between the two modes are expected due to higher resistance factors typically associated with qualitative resistance for QoIs, consequently, resistant and sensitive isolates are clearly distinct.

Azoxystrobin cumulative frequency distributions were significantly different between populations (Figure 2.12; Anderson-Darling K-sample test, AD = 47.3, T.AD = 34.02, $p < 0.001$). A high frequency of sensitive strains was observed for azoxystrobin in the Chile population of *Z. tritici* (Figure 2.12). 80% of the isolates have $\log_{10} EC_{50}$ values under -1.00, similar values to the reference isolate IPO323 ($\log_{10} EC_{50}$ = -1.626) (Table 2.5). In Argentina, Ruta 50 and La Estanzuela, 7.4, 7.5 and 31% of the strains were sensitive, respectively (Figure 2.12). The EC_{50} of the most resistant strains for these populations were greater than in Chile, with resistant factors of 599, 663 and 879, respectively (Table 2.6).

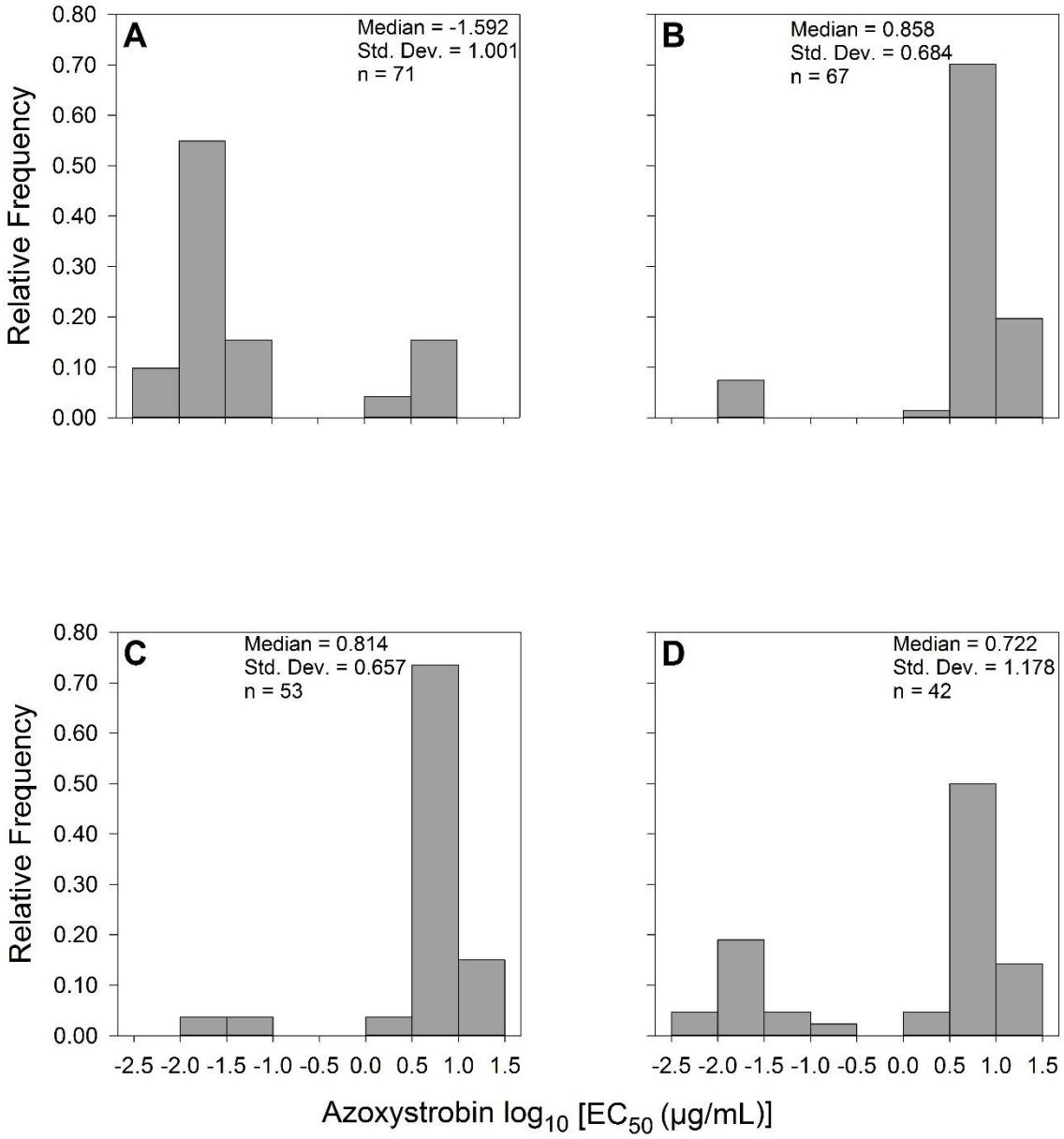

Figure 2.11 Frequency distribution of azoxystrobin log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A), Argentina (B), Ruta 50-Uruguay (C) and La Estanzuela-Uruguay (D).

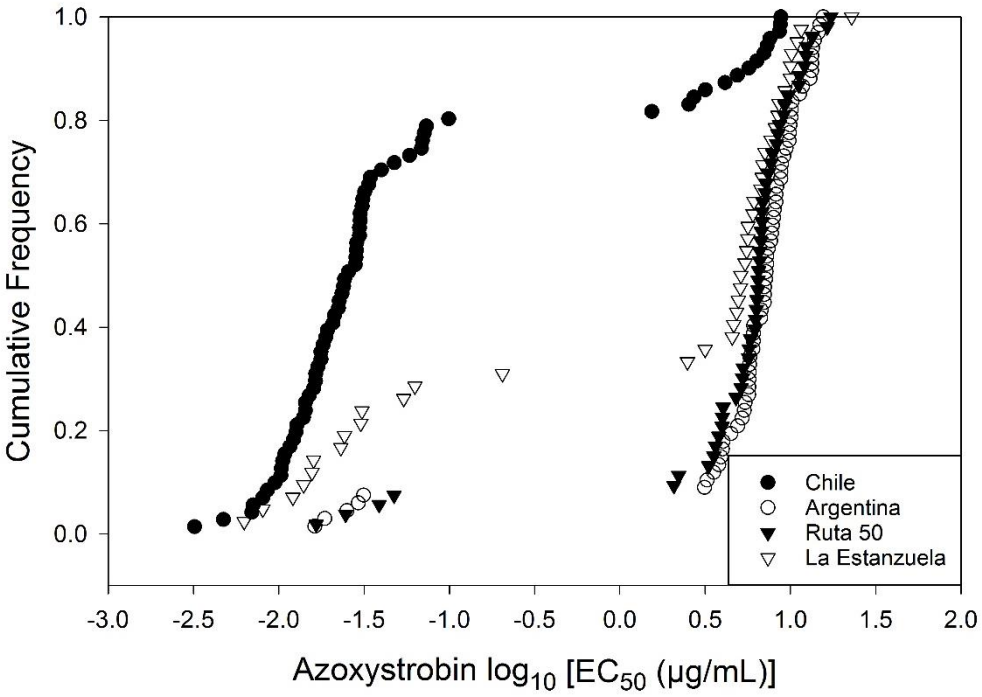

Figure 2.12 Cumulative frequency distribution of azoxystrobin log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53) and La Estanzuela-Uruguay (n=42).

Table 2.5 QoI and multisite sensitivities of South American *Zymoseptoria tritici* field populations from Chile, Argentina, Ruta 50-UY and La Estanzuela-UY.

Population	n	Azoxystrobin			Chlorothalonil		
		Log_{10} EC_{50}±SE[1] (mean)	SD[2]	EC_{50}[3] (µg/mL)	Log_{10} EC_{50}±SE[1] (mean)	SD[2]	EC_{50}[3] (µg/mL)
Chile	71	-1.221 ± 0.119	1.001	0.060	-0.865 ± 0.029	0.250	0.136
Argentina	67	0.685 ± 0.084	0.684	4.842	-0.765 ± 0.031	0.256	0.172
Ruta 50	53	0.644 ± 0.090	0.657	4.539	-0.677 ± 0.035	0.255	0.210
La Estanzuela	42	0.069 ± 0.182	1.178	1.172	-0.809 ± 0.047	0.303	0.155
IPO323[4]	20	-1.626 ± 0.041	-	-	-0.914 ± 0.031	-	-

[1]EC_{50} are the mean of two replicates for each isolate.
[2]SD are in log scale.
[3]EC_{50} are the back transformation of Log_{10} EC_{50}.
[4]EC_{50} are the mean of 19 independent experiments.

Table 2.6 Range of QoI, multisite and SDHI fungicides resistance factors of South American *Zymoseptoria tritici* field populations from Chile, Argentina, Ruta 50-UY and La Estanzuela-UY.

Population	n	Azoxystrobin (Range of RF)[1]	Chlorothalonil (Range of RF)[1]	Bixafen (Range of RF)[1]	Fluopyram (Range of RF)[1]
Chile	71	0.12 - 338	0.23 - 2.67	0.22 - 6.60	0.03 - 5.90
Argentina	67	0.63 - 599	0.33 - 3.69	0.25 - 7.93	0.28 - 23.0
Ruta 50	53	0.64 - 663	0.48 - 5.44	0.08 - 10.5	0.31 - 12.4
La Estanzuela	42	0.24 - 879	0.20 - 5.26	0.13 - 14.4	0.52 - 16.8

[1]Resistance factor was calculated by dividing absolute values of the lowest EC_{50} and the highest EC_{50} in the range by IPO323 EC_{50}.

2.3.1.4 Sensitivity profiles to chlorothalonil

The mean EC_{50} values for all four populations were slightly higher than the reference isolate IPO323, which had an average $\log_{10}$ EC_{50} of -0.914 to chlorothalonil (Table 2.5). The relative frequency distributions of $\log_{10}$ EC_{50} values were unimodal for all South American populations (Figure 2.13). Resistance factors were similar between locations, varying from 0.23 to 2.67, 0.33 to 3.69, 0.48 to 5.44 and 0.20 to 5.26 for Chile, Argentina, Ruta 50 and La Estanzuela, respectively (Table 2.6).

The K-sample Anderson-Darling tested cumulative frequency distributions and found significant differences between populations (Anderson-Darling K-sample test, AD = 8.12, T.AD = 3.93, p = 0.004). The cumulative frequency curves for chlorothalonil sensitivity from Chile and Ruta 50 are the only ones which are distinctly different (Figure 2.14). Isolates from Chile tended to be more sensitive than those sampled in Ruta 50 (Kolmogorov-Smirnov two-sample test, D=0.29, $\chi 2$ approximation = 10.1, 2 df, p = 0.006).

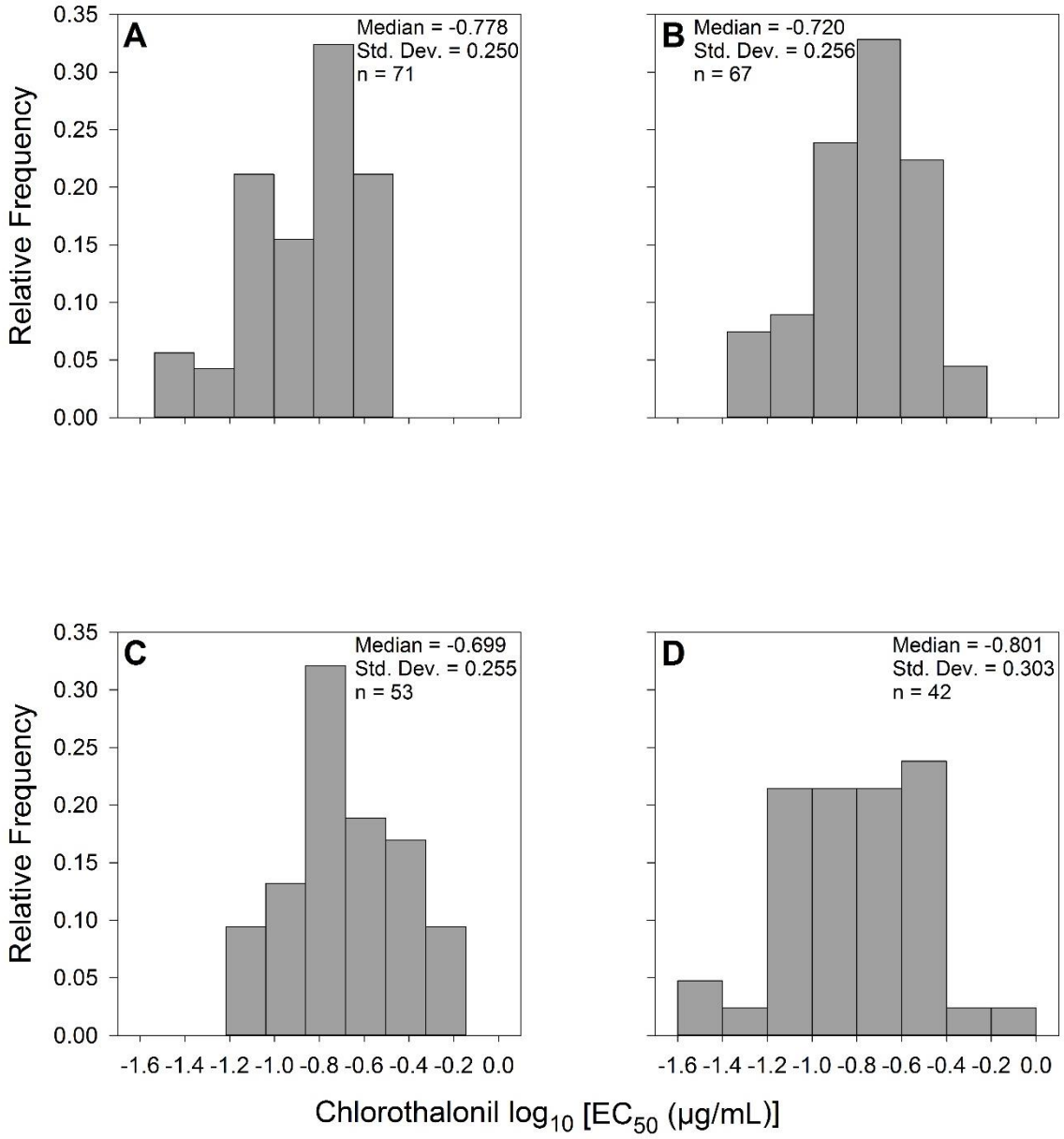

Figure 2.13 Frequency distribution of chlorothalonil log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A), Argentina (B), Ruta 50-Uruguay (C) and La Estanzuela-Uruguay (D).

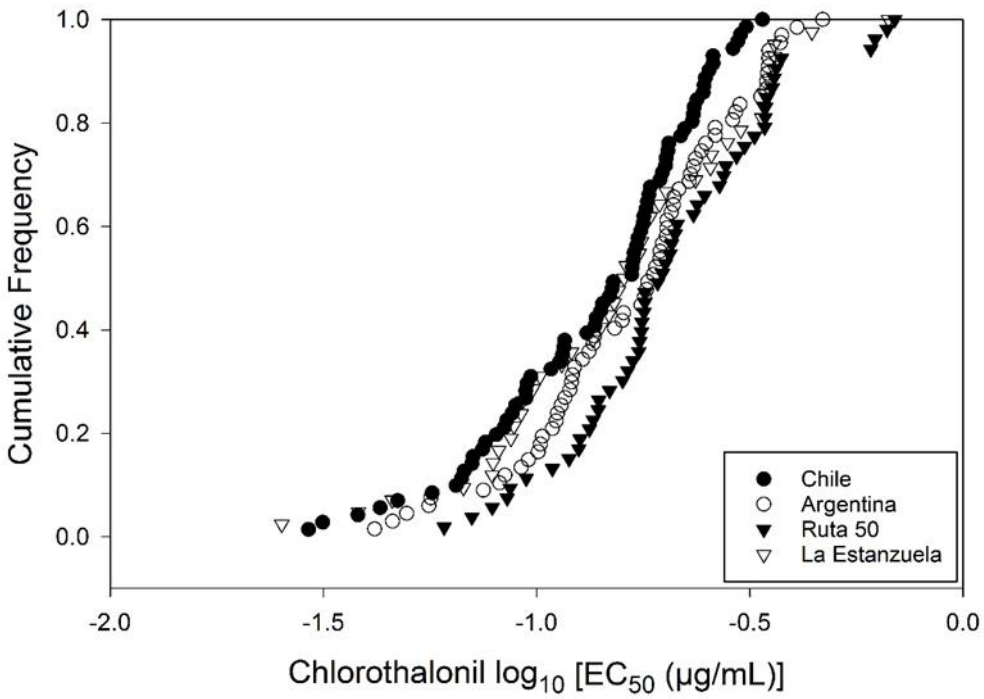

Figure 2.14 Cumulative frequency distribution of chlorothalonil log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53) and La Estanzuela-Uruguay (n=42).

2.3.1.5 Sensitivity profiles of SDHI fungicides

The distribution of log EC_{50} for bixafen (Figure 2.15) and fluopyram (Figure 2.16) was unimodal for all four South American populations. Prior to the introduction of a new fungicide mode of action, the baseline sensitivity of a population is expected to have a resistance factor variation of 10 to 100 between the least and the most sensitive isolates (Birchmore *et al.*, 1996). Bixafen and fluopyram resistant factors in isolates from Chile, Argentina, Ruta 50 and La Estanzuela ranged up to 6.60 – 5.90 (bixafen – fluopyram), 7.93 – 23.0, 10.5 – 12.4 and 14.4 – 16.8 compared to IPO323, respectively (Table 2.6). Correspondingly, the mean log_{10} EC_{50} values for each population was resembled that of IPO323 (bixafen log_{10} EC_{50} = -1.405, fluopyram log_{10} EC_{50} = -1.003) (Table 2.7).

South American populations of *Z. tritici* had different cumulative frequencies of sensitivity to bixafen (Anderson-Darling K-sample test, AD = 7.99, T.AD = 3.83, p = 0.005). The population from Chile was more sensitive than the one from Argentina (Kolmogorov-Smirnov two-sample test, D=0.33, $\chi 2$ approximation = 14.9, 2 df, $p < 0.001$), with the Uruguayan populations showing signs of bimodality

(Figure 2.17). However, comparisons other than between Chile and Argentina were not significantly different (Kolmogorov-Smirnoff tests P > 0.01).

The distribution of fluopyram sensitivity was different between populations (Anderson-Darling K-sample test, AD = 16.07, T.AD = 10.04, $p < 0.001$). The frequency of isolates with greater fluopyram EC_{50} values was higher in the collections from Argentina, Ruta 50 and La Estanzuela than from Chile (Figure 2.18; Kolmogorov-Smirnov two sample tests: Chile-Argentina, D=0.35, $\chi 2$ approximation = 16.6, 2 df, $p < 0.001$; Chile-Ruta 50, D=0.36, $\chi 2$ approximation = 16.1, 2 df, $p < 0.001$; Chile-La Estanzuela, D=0.36, $\chi 2$ approximation = 13.8, 2 df, $p < 0.001$).

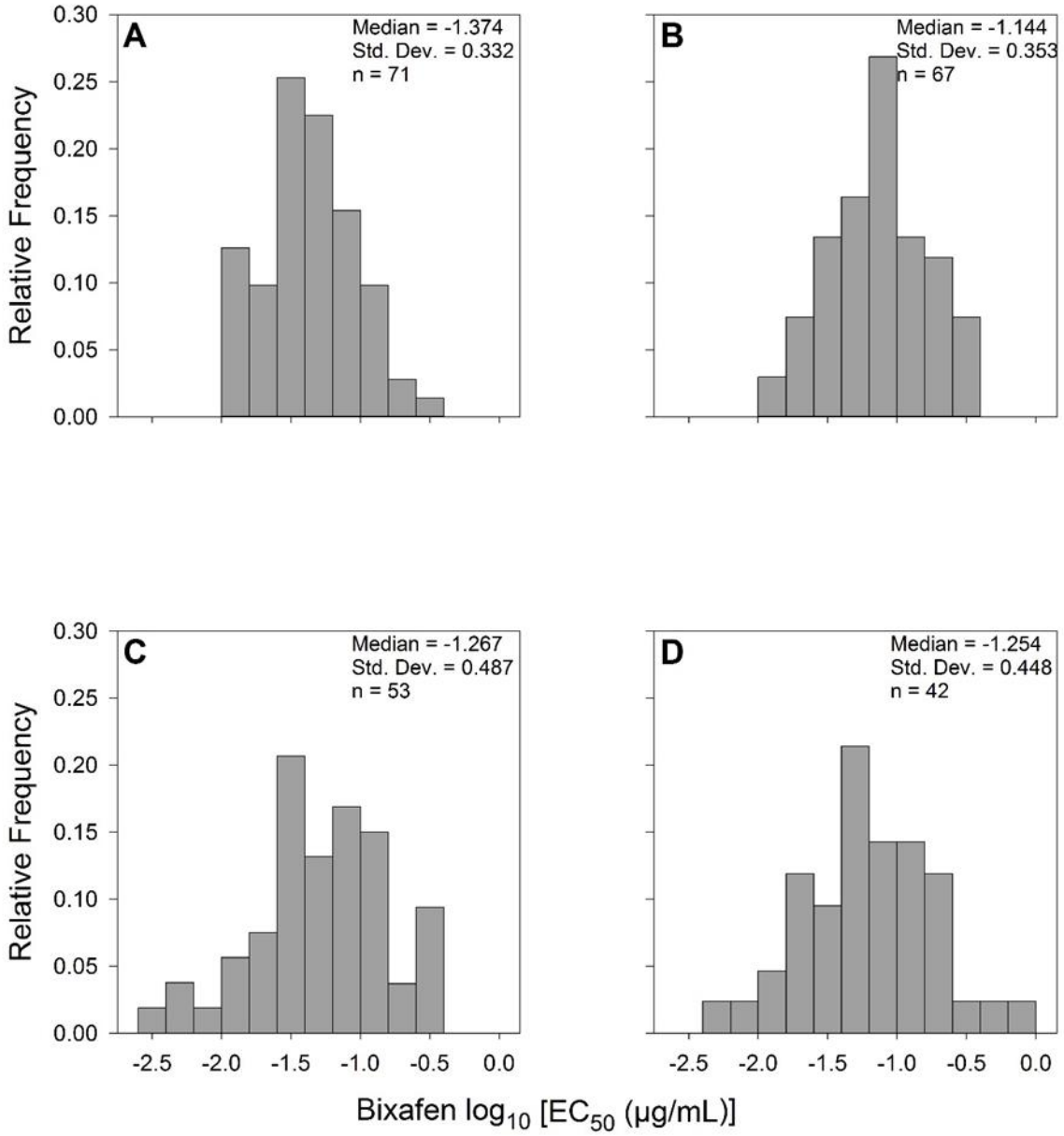

Figure 2.15 Frequency distribution of bixafen log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A), Argentina (B), Ruta 50-Uruguay (C) and La Estanzuela-Uruguay (D).

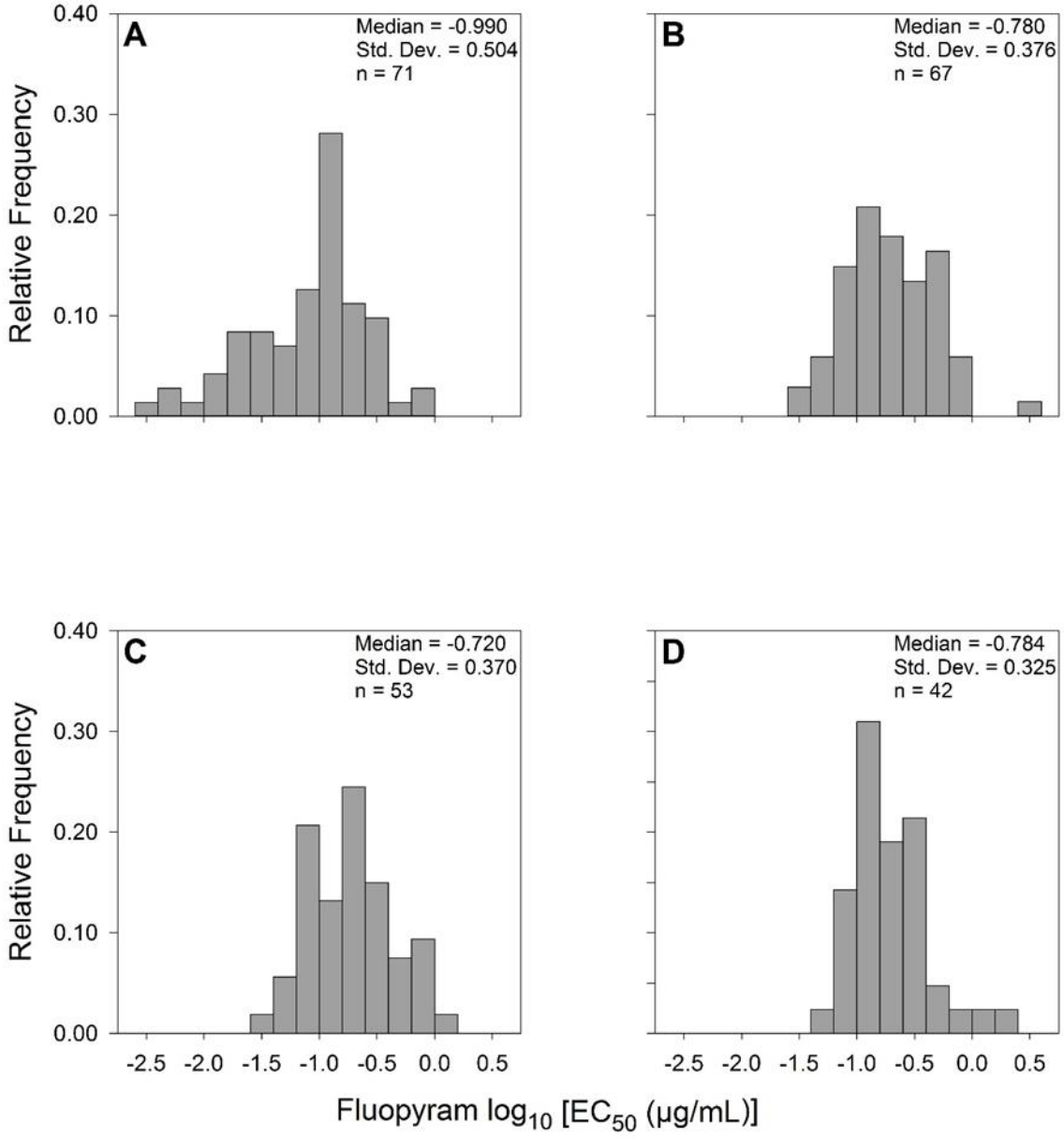

Figure 2.16 Frequency distribution of fluopyram log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A), Argentina (B), Ruta 50-Uruguay (C) and La Estanzuela-Uruguay (D).

Table 2.7 SDHI sensitivities of South American *Zymoseptoria tritici* field populations from Chile, Argentina, Ruta 50-UY and La Estanzuela-UY.

Population	n	Bixafen			Fluopyram		
		Log_{10} EC_{50}±SE[1] (mean)	SD[2]	EC_{50}[3] (µg/mL)	Log_{10} EC_{50}±SE[1] (mean)	SD[2]	EC_{50}[3] (µg/mL)
Chile	71	-1.366 ± 0.039	0.332	0.043	-1.102 ± 0.059	0.504	0.079
Argentina	67	-1.138 ± 0.043	0.353	0.073	-0.717 ± 0.046	0.376	0.192
Ruta 50	53	-1.272 ± 0.067	0.487	0.053	-0.732 ± 0.051	0.370	0.185
La Estanzuela	42	-1.225 ± 0.069	0.448	0.059	-0.715 ± 0.050	0.325	0.193
IPO323[4]	20	-1.405 ± 0.064	-	-	-1.003 ± 0.069	-	-

[1]EC_{50} are the mean of two replicates for each isolate.
[2]SD are in log scale.
[3]EC_{50} are the back transformation of Log_{10} EC_{50}.
[4]EC_{50} are the mean of 19 independent experiments.

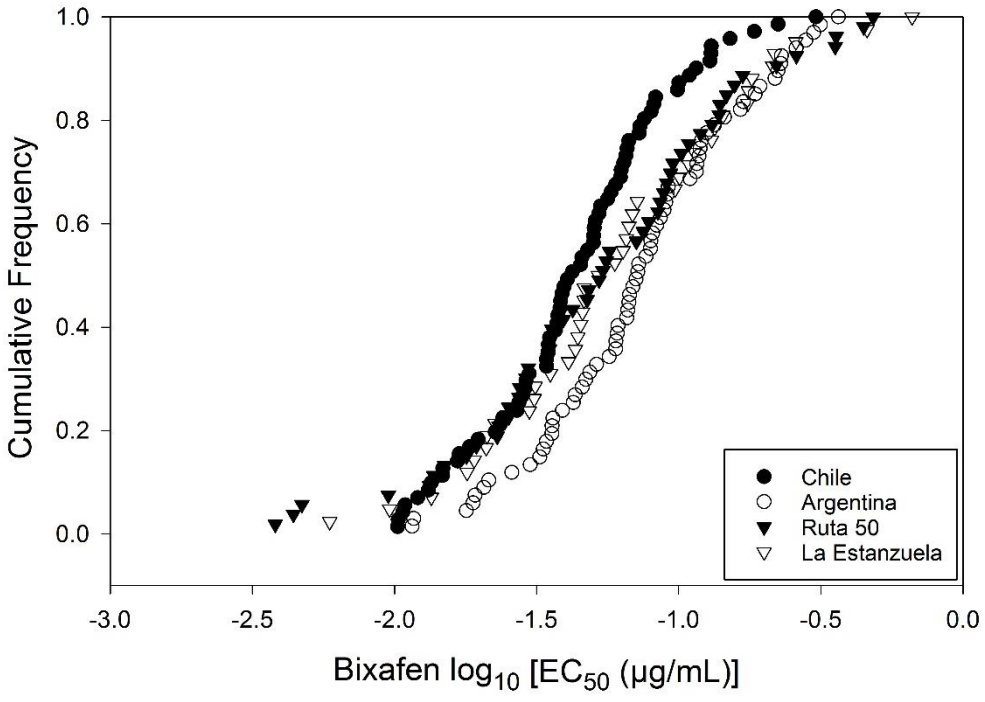

Figure 2.17 Cumulative frequency distribution of bixafen log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53) and La Estanzuela-Uruguay (n=42).

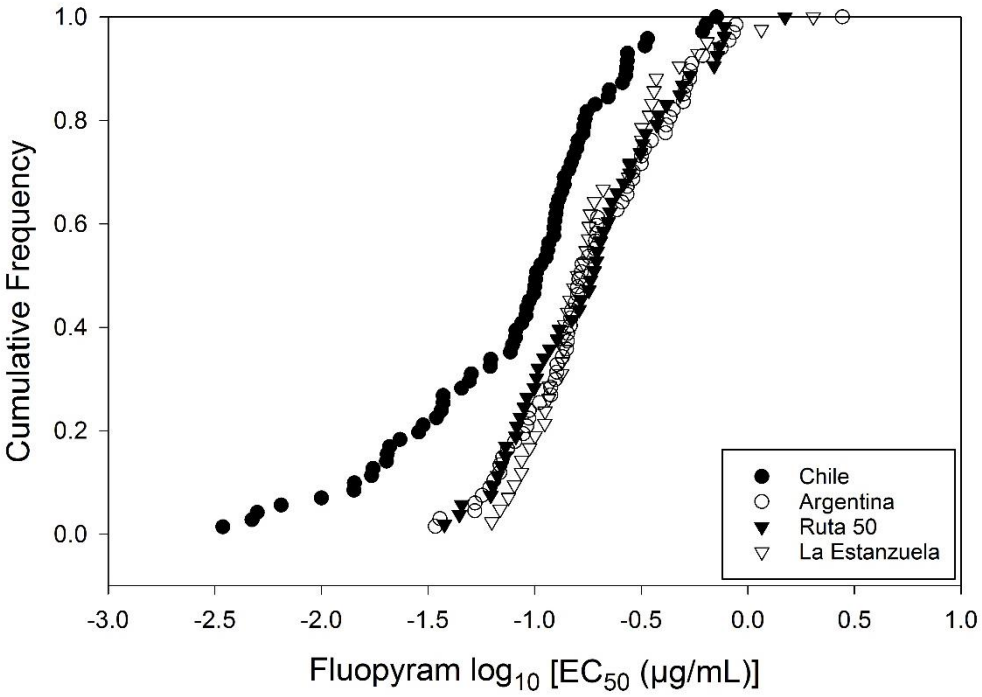

Figure 2.18 Cumulative frequency distribution of fluopyram log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53) and La Estanzuela-Uruguay (n=42).

2.3.1.6 Cross-resistance of SDHI fungicides

There was a significant relationship between the pair of SDHI fungicides for all four populations of *Z. tritici* taken from South America (Figure 2.19). Correlations between bixafen and fluopyram EC_{50} values of strains from Chile (A), Argentina (B), Ruta 50 (C) and La Estanzuela (D) were 0.56, 0.80, 0.91 and 0.89 correspondingly ($p < 0.001$ in all cases, n = 71, 67, 53, 42 respectively). A few isolates from Argentina and Ruta 50 were very insensitive for fluopyram (EC_{50} values over 1 µg/mL) whereas the sensitivity for bixafen was not reduced in these isolates.

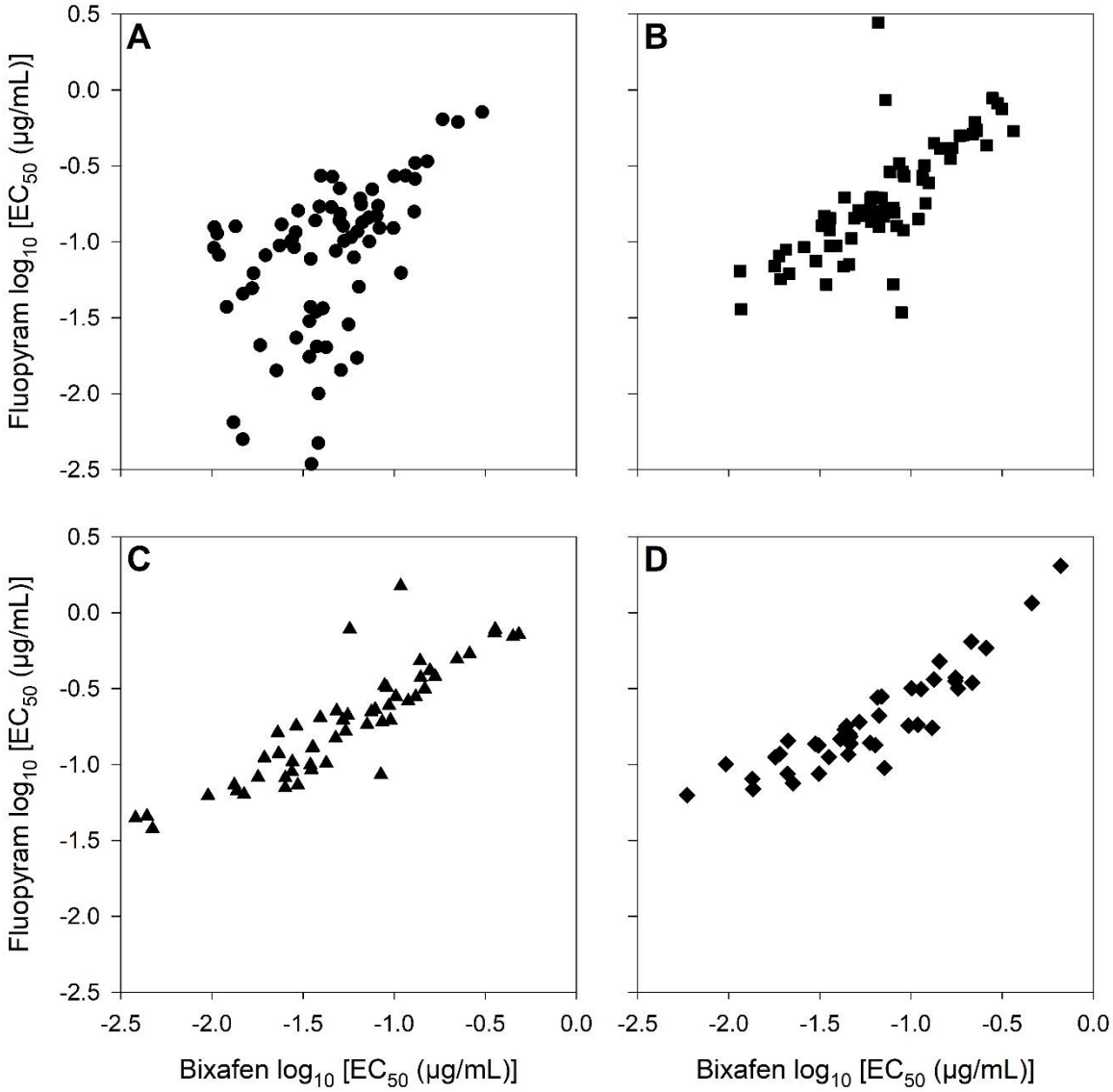

Figure 2.19 Correlation between bixafen and fluopyram log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (A, n=71), Argentina (B, n=67), Ruta 50-Uruguay (C, n=53) and La Estanzuela-Uruguay (D, n=42).

2.3.1.7 Cross-resistance of different fungicide classes

Cross-resistance between the different classes of fungicides was tested for all South American isolates collected. There was no correlation between any of the azole fungicides and chlorothalonil (Figure 2.20; epoxiconazole-chlorothalonil, r = 0.005, p = 0.94, n = 233; tebuconazole-chlorothalonil, r = 0.001, p = 0.98, n = 233; prochloraz-chlorothalonil, r = 0.04, p = 0.57, n = 233).

SDHI and azole log_{10} EC_{50} values were uncorrelated for South American *Z. tritici* isolates, independent of the population to which they belong (Figure 2.21; epoxiconazole-bixafen, r = 0.02, p = 0.75, n = 233; tebuconazole-bixafen, r = -0.05, p = 0.41, n = 233; prochloraz-bixafen, r = 0.07, p = 0.28, n = 233/Figure 2.22; epoxiconazole-fluopyram, r = -0.01, p = 0.82, n = 233; tebuconazole-fluopyram, r = -0.06, p = 0.36, n = 233; prochloraz-fluopyram, r = 0.04, p = 0.50, n = 233).

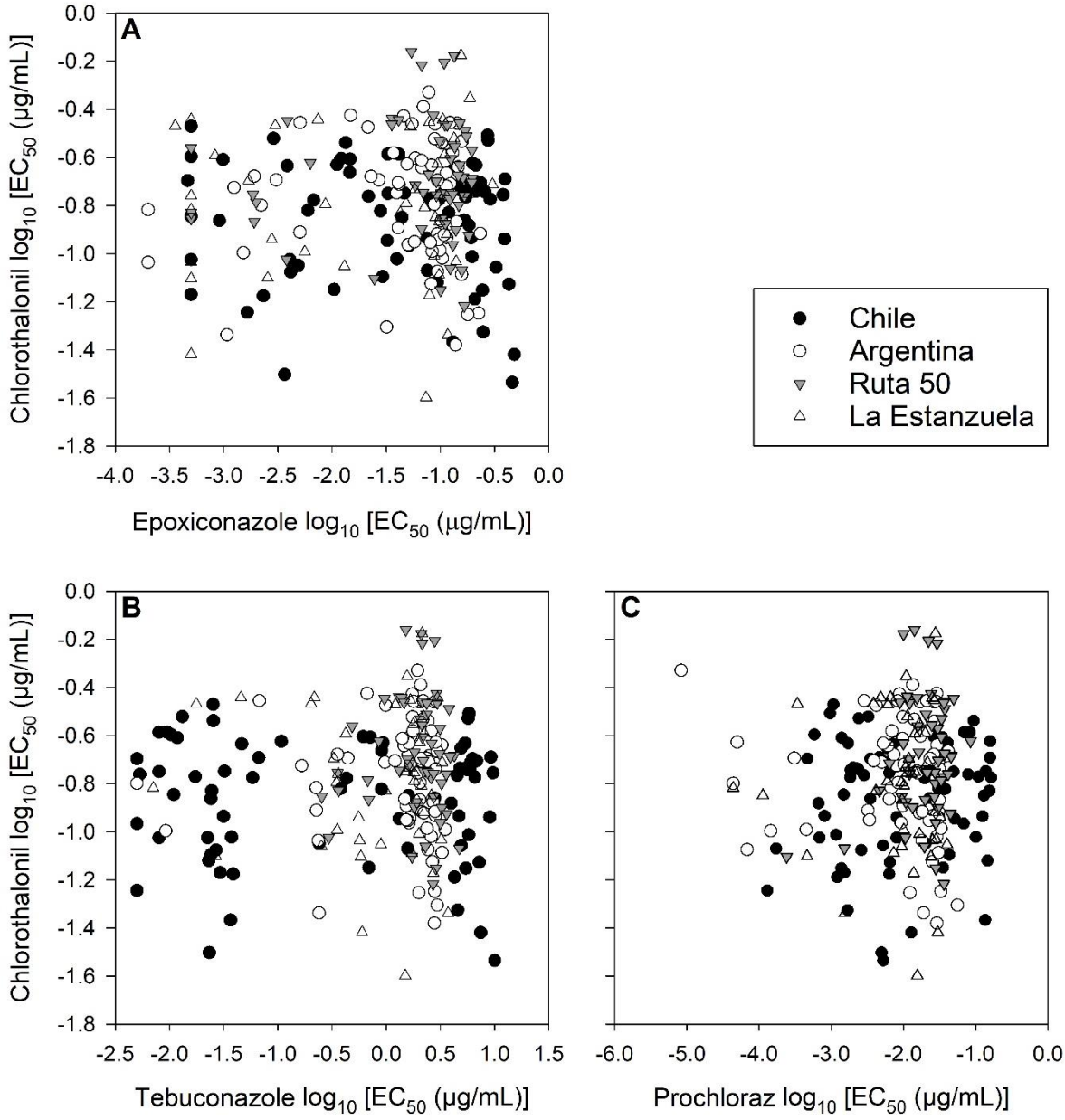

Figure 2.20 Correlation between azoles and chlorothalonil log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53) and La Estanzuela-Uruguay (n=42).

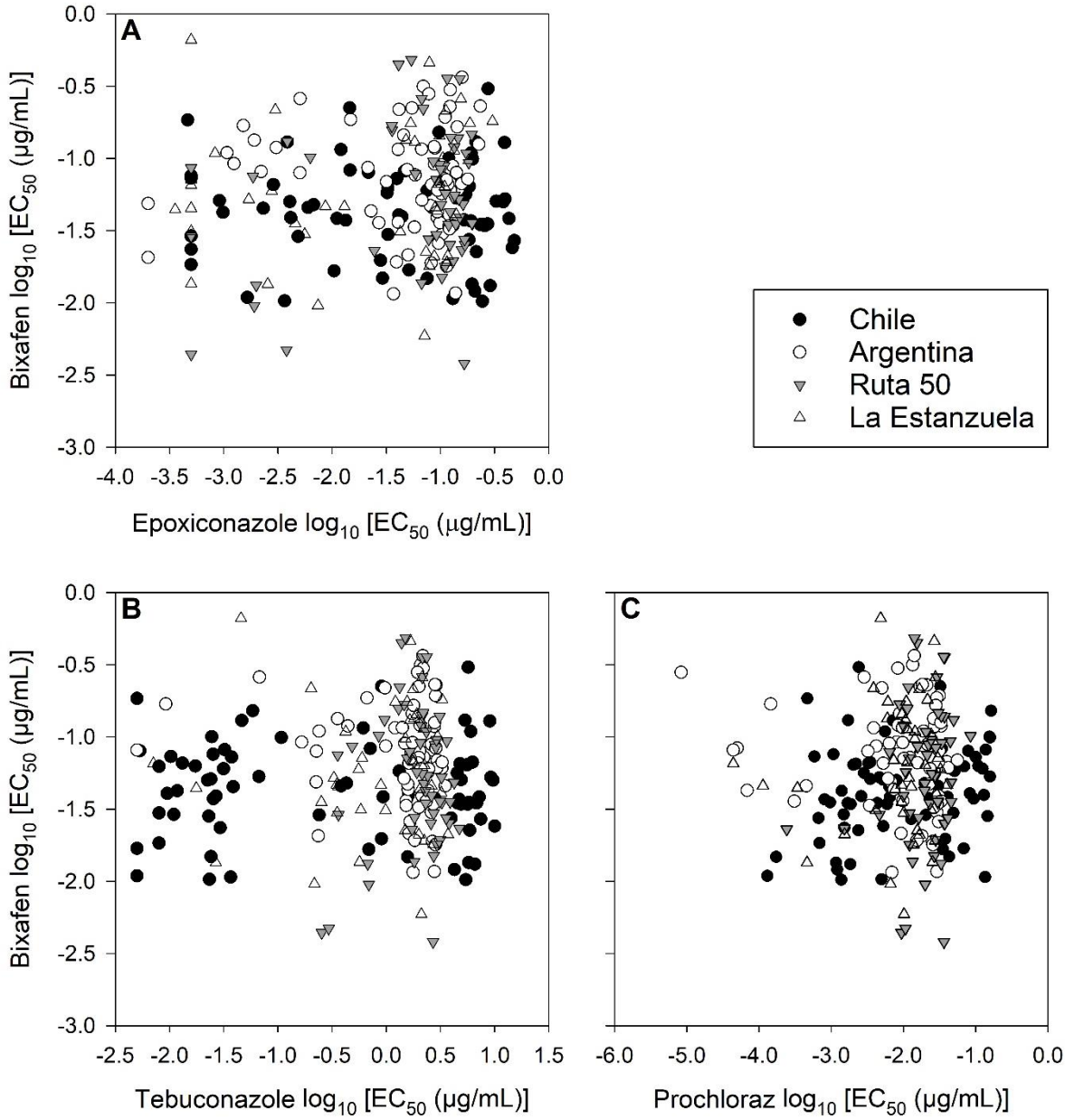

Figure 2.21 Correlation between azoles and bixafen log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53) and La Estanzuela-Uruguay (n=42).

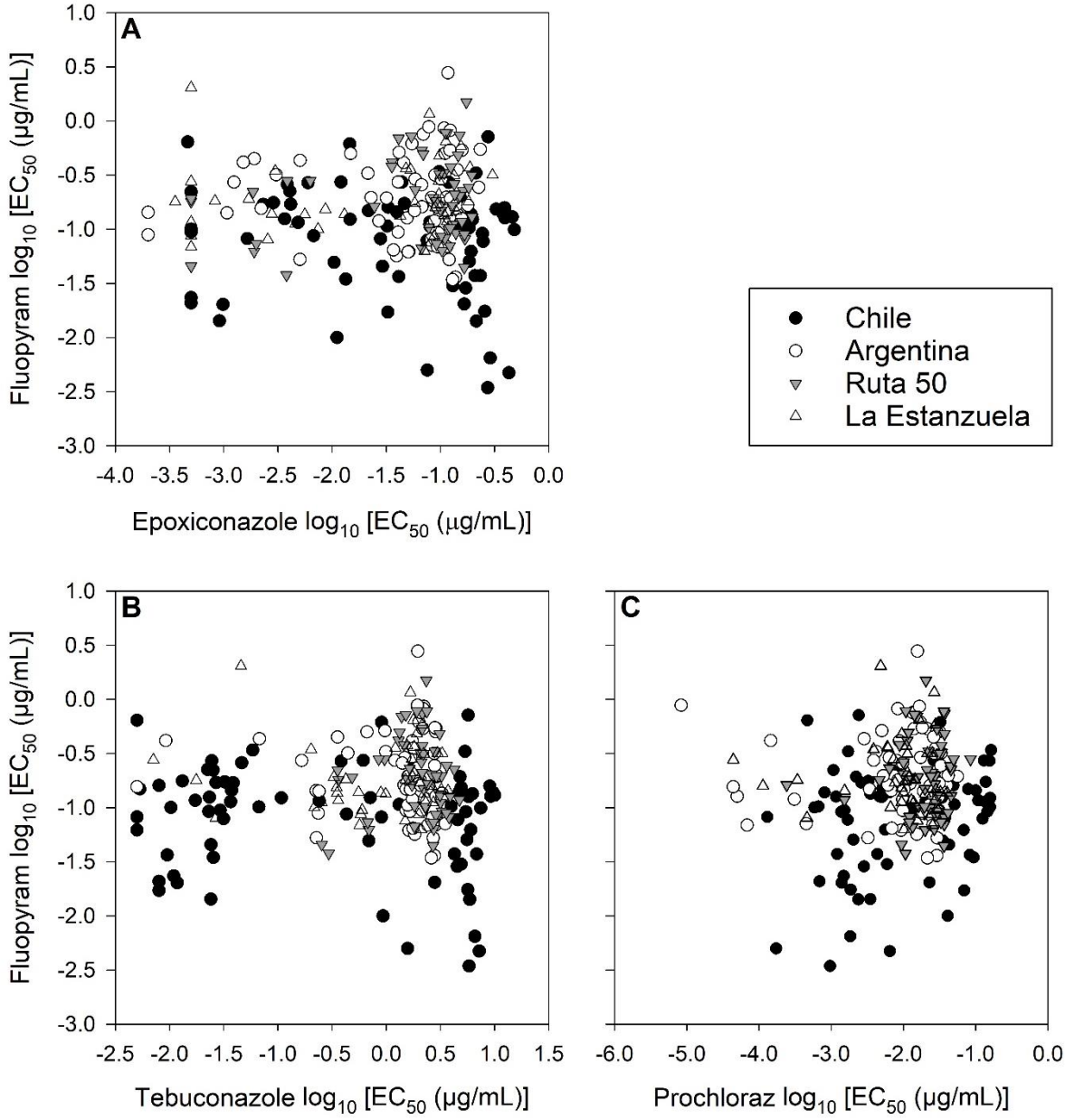

Figure 2.22 Correlation between azoles and fluopyram log_{10} EC_{50} values of South American *Zymoseptoria tritici* field populations from Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53) and La Estanzuela-Uruguay (n=42).

To test cross-resistance between QoIs and other fungicide classes the South American isolates were categorized as sensitive [log_{10} EC_{50} < 0 (EC_{50} < 1 ppm)] or resistant [log_{10} EC_{50} ≥ 0 (EC_{50} ≥ 1 ppm)] to azoxystrobin. Azoxystrobin sensitivity was not correlated with log_{10} EC_{50} values of epoxiconazole (r = -0.18), tebuconazole (r = -0.10) and prochloraz (r = 0.14) for sensitive strains (Figure 2.23; $p > 0.05$ in all cases, n = 79). However, significant positive correlation was found between azoxystrobin and

chlorothalonil (r = 0.31), bixafen (r = 0.49) and fluopyram (r = 0.7) for the same category of QoI sensitive strains (Figure 2.23; $p < 0.005$ in all cases, n = 79).

There was no significant relationship between epoxiconazole (r = 0.04), tebuconazole (r = -0.04) and prochloraz (r = 0.09) with azoxystrobin for QoI insensitive strains (Figure 2.24; $p > 0.05$ in all cases, n = 154). While azoxystrobin was positive correlated to chlorothalonil (r = 0.62), bixafen (r = 0.64) and fluopyram (r = 0.55) (Figure 2.24; $p < 0.001$ in all cases, n = 154).

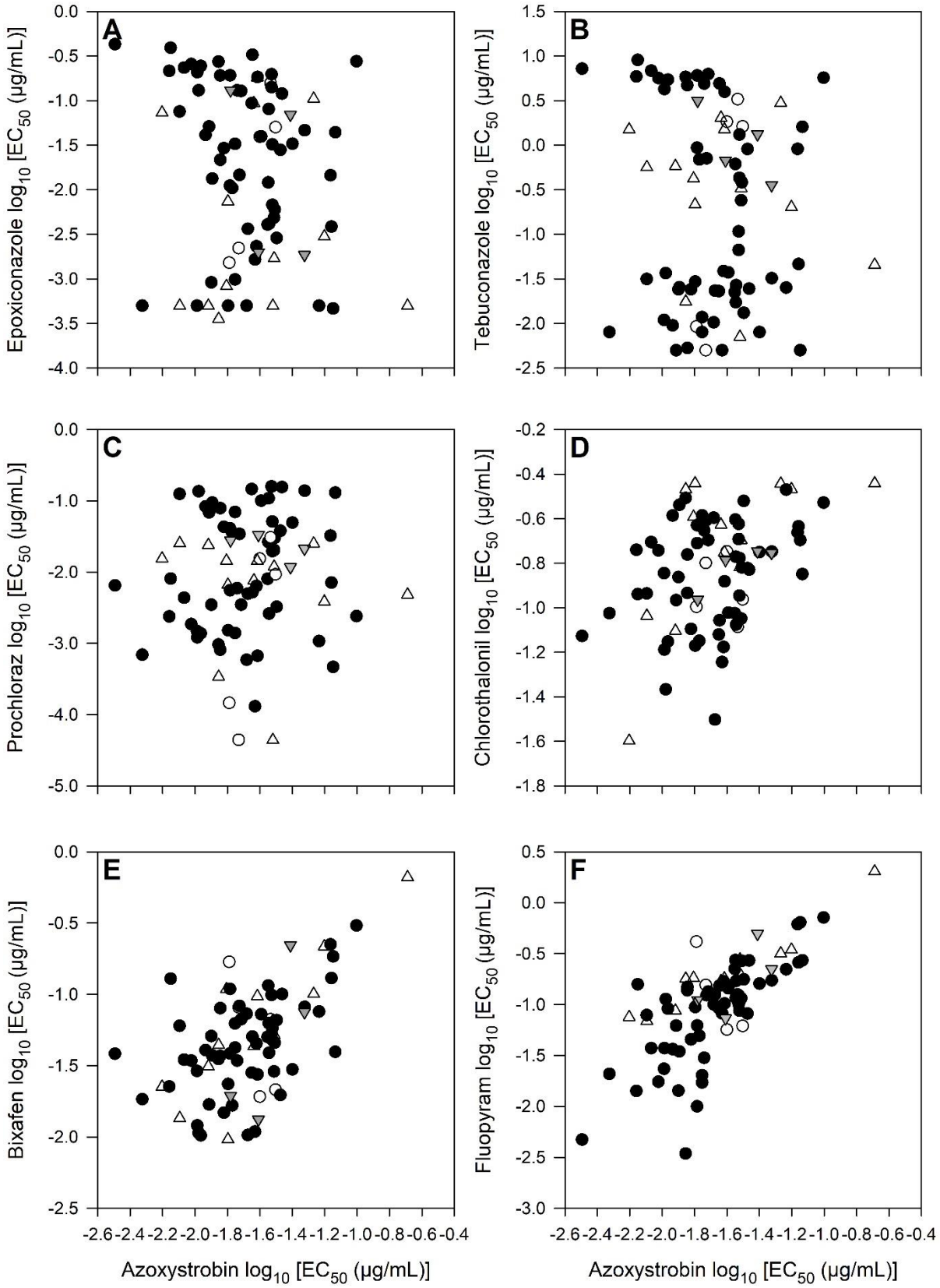

Figure 2.23 Correlation between azoles (A, B, C), chlorothalonil (D) and SDHI fungicides (E, F) with azoxystrobin sensitive isolates from Chile (Black circle, n=57), Argentina (White circle, n=5), Ruta 50-Uruguay (Triangle down, n=4) and La Estanzuela-Uruguay (Triangle up, n=13).

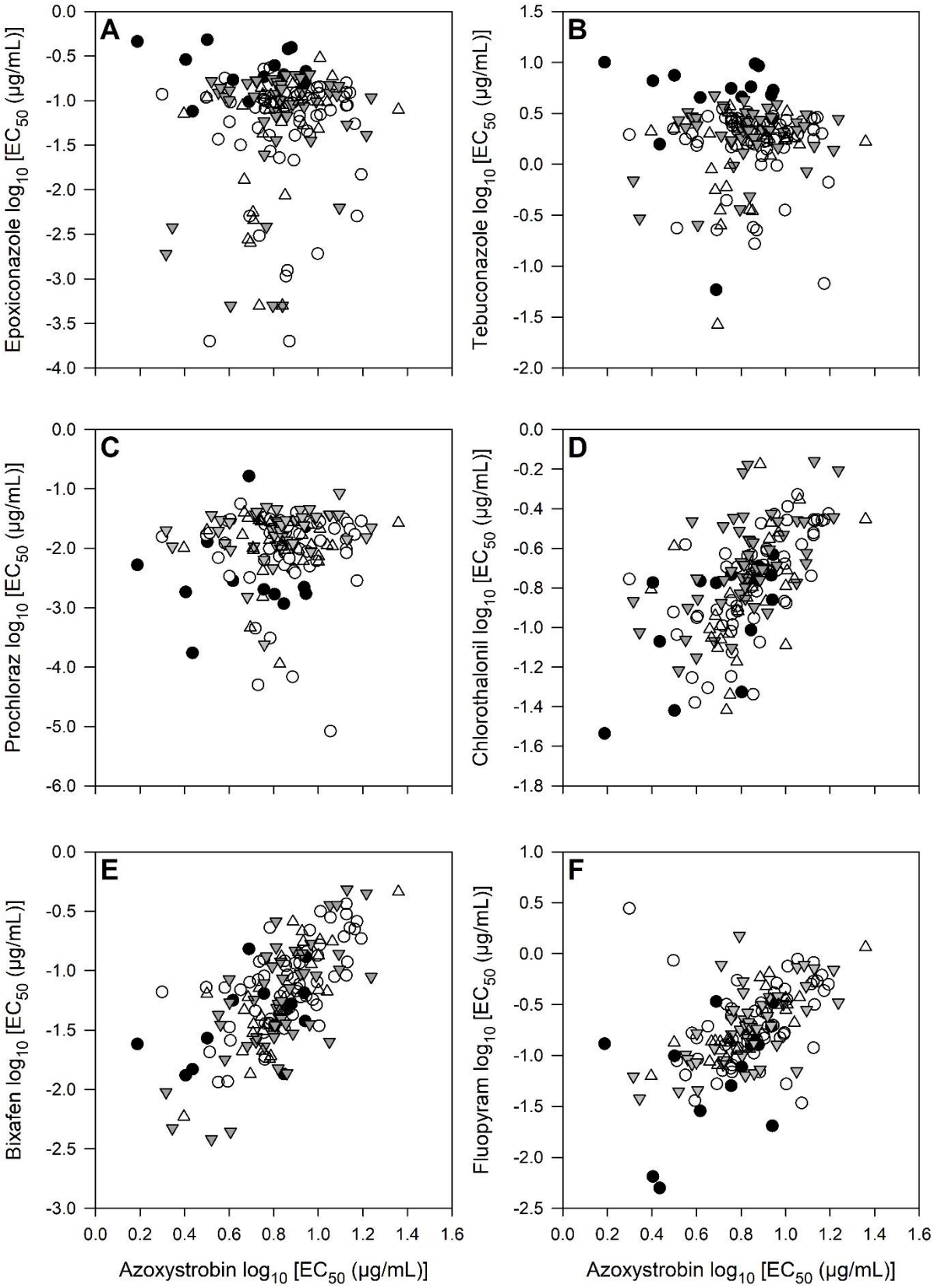

Figure 2.24 Correlation between azoles (A, B, C), chlorothalonil (D) and SDHI fungicides (E, F) with azoxystrobin resistant isolates from Chile (Black circle, n=14), Argentina (White circle, n=62), Ruta 50-Uruguay (Triangle down, n=49) and La Estanzuela-Uruguay (Triangle up, n=29).

2.3.1.8 Sensitivity of *Zymoseptoria tritici* field isolates to tolnaftate and carbendazim

A preliminary experiment was carried out to determine single doses of tolnaftate and carbendazim that could be used to assess *Z. tritici* field isolates regarding their phenotypes for Multiple Drug Resistance (MDR) and Methyl Benzimidazole Carbamates (MBCs) resistance, respectively. This first assay tested six strains from Oregon and four reference isolates (LN591, IRE30, IPO323 and NT321.17) against 0, 1.6E-3, 5E-3, 1.5E-2, 4.5E-2, 1.4E-1, 4.1E-1, 1.23, 3.70, 11.11, 33.33 and 100 µg/mL of tolnaftate and 0, 1.4E-4, 5E-4, 2E-3, 7E-3, 3E-2, 1E-1, 3.8E-1, 1.42, 5.33, 20 and 75 µg/mL of carbendazim.

The concentrations of 33.33 and 100 µg/mL of tolnaftate did not dilute properly in the medium and no absorbance could be measured due to insolubility at these concentrations. The reference isolate NT321.17 was the only one to have mycelial growth above the concentration of 10 µg/mL of tolnaftate due to its efflux pump overexpression (Figure 2.25).

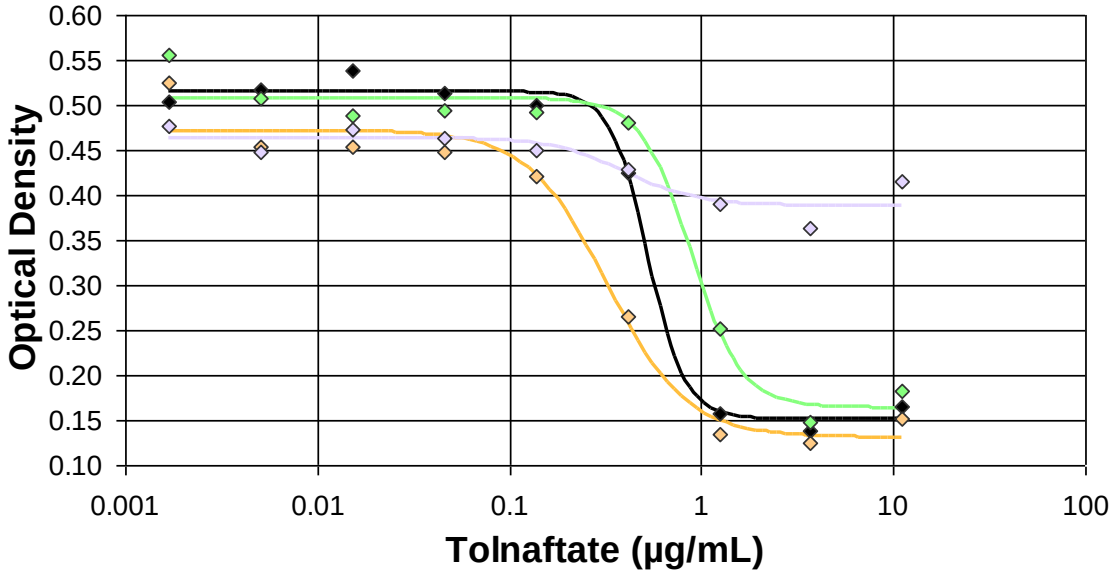

Figure 2.25 Dose-response of fungal growth against tolnaftate concentrations (µg/mL) for strains ORE16.05, ORE16.06, LN591 and NT321.17. ORE16.05: Black; ORE16.06: Green; LN591: Orange; NT321.17: Violet.

Fungal growth could not be measured at the concentration of 75 µg/mL for carbendazim due to fungicide insolubility. Reference strains NT321.17 and LN591 grew in all fungicide dilutions. These two strains are known for carrying the point mutation E198A in the β-tubulin encoding gene, while isolates

with the wild type tubulin did not show any growth in concentrations above 0.1 µg/mL of carbendazim (Figure 2.26). Based in this preliminary assay the concentrations of 10 µg/mL of tolnaftate and 1.0 µg/mL of carbendazim were chosen as discriminatory doses for characterizing the field isolates as MDR and MBC resistant phenotypes, respectively.

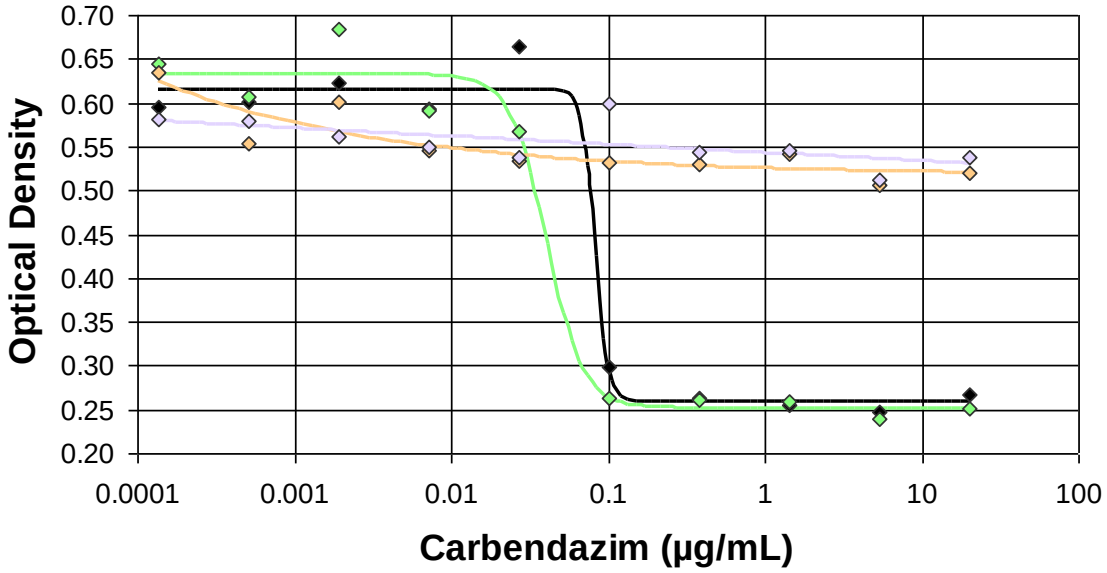

Figure 2.26 Dose-response of fungal growth against carbendazim concentrations (µg/mL) for strains ORE16.05, ORE16.06, LN591 and NT321.17. ORE16.05: Black; ORE16.06: Green; LN591: Orange; NT321.17: Violet.

None of the South American *Z. tritici* field populations had strains with this MDR phenotype. The MBC resistance phenotype was detected in all four populations. For Chile, Argentina, Ruta 50 and La Estanzuela populations, 75, 43, 77 and 26% of the isolates showed growth at 1.0 µg/mL of carbendazim, respectively (Table 2.8).

Table 2.8 Frequency of MDR and MBC resistance in *Zymoseptoria tritici* field populations from Chile, Argentina, Ruta 50-UY and La Estanzuela-UY.

Population	n	Tolnaftate (10 µg/mL)[1]		Carbendazim (1.0 µg/mL)[1]	
		Sensitive strains	Resistance (%)	Sensitive strains	Resistance (%)
Chile	71	71	0	18	75
Argentina	67	67	0	38	43
Ruta 50	53	53	0	12	77
La Estanzuela	42	42	0	31	26

[1]Fungal growth was measured in two replicates.

2.3.2 Fungicide sensitivity in global *Zymoseptoria tritici* field populations

Fungicide sensitivity (log_{10} EC_{50} values) of *Z. tritici* field populations sampled in different continents was assessed and compared to the South American populations described above. The populations were grouped as Reference (Rothamsted-UK), South America (Chile, Argentina, Ruta 50-UY and La Estanzuela-UY), North America (Oregon-USA and Michigan-USA), Eastern Europe (Czech Republic and Slovakia) and New Zealand.

2.3.2.1 Azole fungicides

Azole sensitivities of 522 single pycnidial isolates were determined for epoxiconazole, tebuconazole and prochloraz, with two technical replicates per isolate. The combined ANOVA showed that sensitivity behaved differently across the groups for each azole ($p < 0.001$), consequently, the three fungicides were analysed separately. Furthermore, there were significant differences between populations within groups ($p < 0.001$) for all azoles (Table 2.9).

Table 2.9 ANOVA in the predicted means of azole sensitivities for groups.

	Epoxiconazole		Tebuconazole		Prochloraz	
	Log_{10} EC_{50}±SE	P	Log_{10} EC_{50}±SE	P	Log_{10} EC_{50}±SE	P
Group	-	<0.001	-	<0.001	-	<0.001
Rothamsted	-0.087±0.08	-	0.362±0.08	-	-0.841±0.08	-
South America[1]	-1.434±0.04	0.003	-0.080±0.04	<0.001	-1.985±0.04	0.01
North America[2]	-2.022±0.06	0.015	-0.467±0.06	0.284	-1.880±0.06	0.749
Eastern Europe[3]	-0.923±0.06	0.244	0.309±0.06	0.822	-1.909±0.07	<0.001
New Zealand	-0.681±0.08	-	0.653±0.08	-	-2.611±0.09	-
LSD (5%)	0.194	-	0.190	-	0.198	-

[1]South America: Chile, Argentina, Ruta 50 and La Estanzuela.
[2]North America: Michigan and Oregon.
[3]Eastern Europe: Slovakia and Czech Republic.

ANOVA also demonstrated significative differences between the South American populations for all fungicides. Although, within the other groups, significative differences were only detected in North America to epoxiconazole and in Eastern Europe to prochloraz (Table 2.9).

The reference population from Rothamsted had higher mean log_{10} EC_{50} values to epoxiconazole and prochloraz than any other population. On the other hand, New Zealand had a higher mean for tebuconazole, whereas Rothamsted did not differ from Ruta50, Slovakia and Czech Republic (Table 2.10).

Table 2.10 Azole sensitivities of *Zymoseptoria tritici* field populations from Rothamsted, Chile, Argentina, Ruta 50-UY, La Estanzuela-UY, Michigan-USA, Oregon-USA, Slovakia, Czech Republic and New Zealand.

Population	n	Epoxiconazole		Tebuconazole		Prochloraz	
		Log_{10} EC_{50}±SE[1]	EC_{50}[2] (µg/mL)	Log_{10} EC_{50}±SE[1]	EC_{50}[2] (µg/mL)	Log_{10} EC_{50}±SE[1]	EC_{50}[2] (µg/mL)
Rothamsted	50	-0.087±0.08	0.818	0.362±0.08	2.303	-0.841±0.08	0.144
Chile	71	-1.449±0.07	0.035	-0.511±0.07	0.308	-2.059±0.07	0.009
Argentina	67	-1.354±0.07	0.044	0.120±0.07	1.318	-2.051±0.07	0.009
Ruta 50	53	-1.281±0.08	0.052	0.264±0.08	1.836	-1.726±0.08	0.019
La Estanzuela	42	-1.728±0.09	0.019	-0.102±0.09	0.785	-2.079±0.09	0.008
Michigan	45	-2.190±0.09	0.006	-0.539±0.09	0.289	-1.858±0.09	0.014
Oregon	57	-1.890±0.08	0.013	-0.410±0.08	0.389	-1.898±0.08	0.013
Slovakia	39	-1.008±0.09	0.098	0.325±0.09	2.112	-2.248±0.10	0.006
Czech Republic	47	-0.852±0.09	0.141	0.295±0.08	1.974	-1.627±0.09	0.024
New Zealand	51	-0.681±0.08	0.208	0.653±0.08	4.498	-2.611±0.08	0.002
LSD (5%)	-	0.241	-	0.236	-	0.246	-

[1]EC_{50} are the mean of two replicates for each isolate.
[2]EC_{50} are the back transformation of Log_{10} EC_{50}.

Epoxiconazole sensitivity showed differences between multiple groups. South America was less sensitive than North America but more sensitive than Eastern Europe and New Zealand (Figure 2.27).

Between South American samples, La Estanzuela was more sensitive to epoxiconazole, and there was no difference among the others. In North America, Oregon had a higher mean EC_{50} value than Michigan. Meanwhile, the mean for Michigan was not different from the mean for La Estanzuela. There was no difference between Eastern European populations, but Czech Republic showed similar epoxiconazole sensitivity to New Zealand (Figure 2.27).

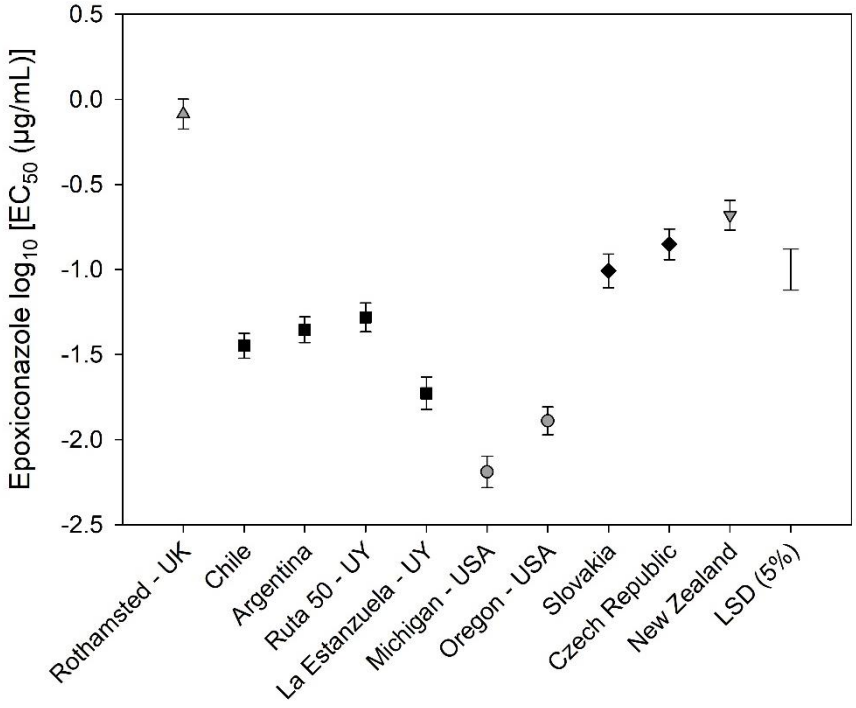

Figure 2.27 Mean of epoxiconazole log_{10} EC_{50} values of *Zymoseptoria tritici* field populations from Rothamsted-UK (n=50), Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53), La Estanzuela-Uruguay (n=42), Michigan-USA (n=45), Oregon-USA (n=57), Slovakia (n=39), Czech Republic (n=47) and New Zealand (n=51). Bars represent the standard error.

In general, as for epoxiconazole, the North American populations were more sensitive to tebuconazole than Eastern European populations, while these were more sensitive than New Zealand. Rothamsted did not differ from Slovakia and Czech Republic in tebuconazole sensitivity (Figure 2.28).

There was a significative difference between South American populations. Chile was the most sensitive to tebuconazole, not differing from Oregon and Michigan. Ruta 50 and Argentina had the higher means of tebuconazole log_{10} EC_{50} value in South America, with the mean of La Estanzuela between these and Chile. Ruta 50 and Argentina means also did not differ from the means of the Eastern European populations (Figure 2.28).

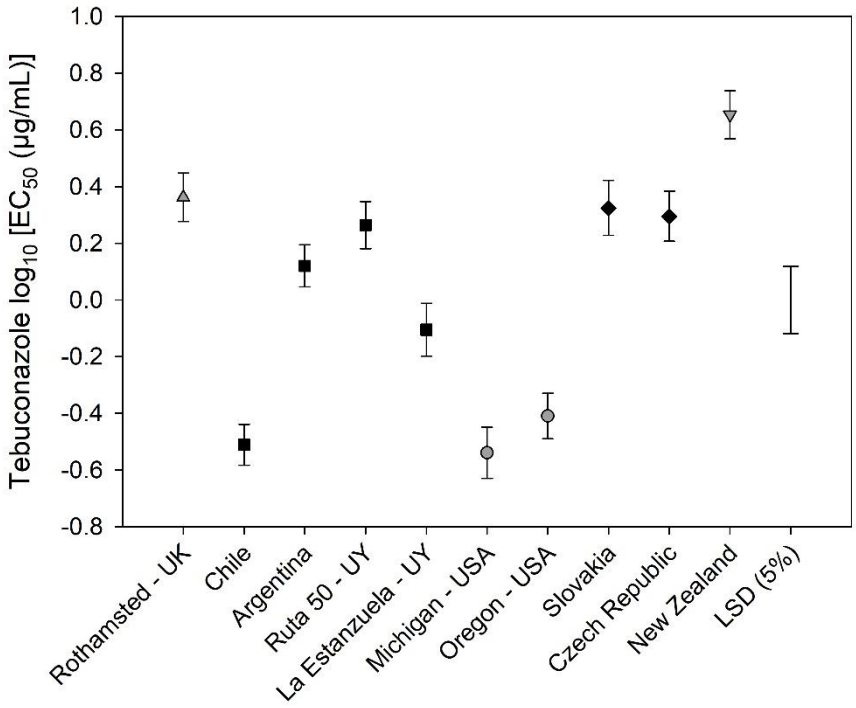

Figure 2.28 Mean of tebuconazole log_{10} EC_{50} values of *Zymoseptoria tritici* field populations from Rothamsted-UK (n=50), Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53), La Estanzuela-Uruguay (n=42), Michigan-USA (n=45), Oregon-USA (n=57), Slovakia (n=39), Czech Republic (n=47) and New Zealand (n=51). Bars represent the standard error.

Prochloraz showed a different trend of sensitivity among the groups and populations. New Zealand had the lowest mean log_{10} EC_{50}. However, no population was as insensitive as the reference from Rothamsted. Slovakia was more sensitive than Czech Republic, but there was no difference between Michigan and Oregon means (Figure 2.29).

In South America, Ruta 50 had the highest mean log_{10} EC_{50} for prochloraz, and there was no difference between the other populations. None of the South American samples differed in prochloraz sensitivity from the North American populations (Figure 2.29).

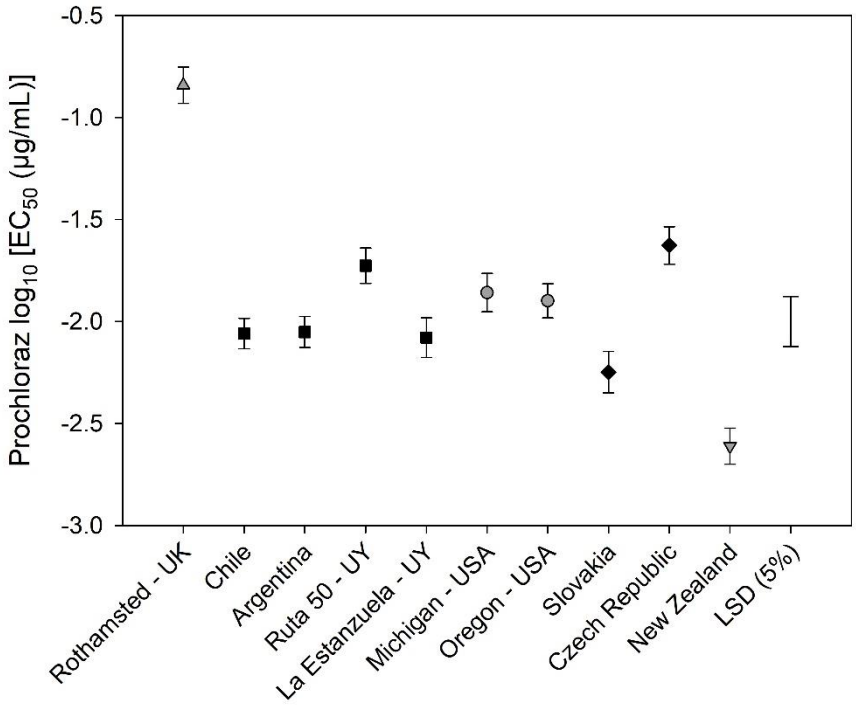

Figure 2.29 Mean of prochloraz log_{10} EC_{50} values of *Zymoseptoria tritici* field populations from Rothamsted-UK (n=50), Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53), La Estanzuela-Uruguay (n=42), Michigan-USA (n=45), Oregon-USA (n=57), Slovakia (n=39), Czech Republic (n=47) and New Zealand (n=51). Bars represent the standard error.

2.3.2.2 Cross-resistance of azole fungicides

Azole cross-resistance was tested to compare with those from South American populations. Epoxiconazole and tebuconazole were positive correlated for all populations, but the degree of cross-resistance between fungicides varied between sites. Michigan ($r = 0.86$, $p < 0.001$, $n = 45$), Oregon ($r = 0.75$, $p < 0.001$, $n = 57$) and New Zealand ($r = 0.69$, $p < 0.001$, $n = 51$) showed strong correlation, meanwhile Rothamsted ($r = 0.37$, $p = 0.008$, $n = 50$), Slovakia ($r = 0.47$, $p = 0.002$, $n = 39$) and Czech Republic ($r = 0.34$, $p = 0.02$, $n = 47$) showed moderate cross-resistance between the pair of fungicides (Figure 2.30).

Strong positive correlation was found between epoxiconazole and prochloraz sensitivities, as for Rothamsted ($r = 0.55$, $p = < 0.001$, $n = 50$), Michigan ($r = 0.82$, $p < 0.001$, $n = 45$), Czech Republic ($r = 0.59$, $p < 0.001$, $n = 47$) and New Zealand ($r = 0.82$, $p < 0.001$, $n = 51$). Weaker but significative positive

correlation was observed for Oregon ($r = 0.41$, $p = 0.002$, $n = 57$) and Slovakia ($r = 0.31$, $p = 0.05$, $n = 39$) (Figure 2.31).

Sensitivity of tebuconazole and prochloraz was not correlated for isolates from Oregon ($r = 0.22$, $p = 0.10$, $n = 57$) or Czech Republic ($r = -0.06$, $p = 0.69$, $n = 47$). Tebuconazole sensitivity tended to decrease as prochloraz sensitivity increased in isolates sampled in Slovakia ($r = -0.44$, $p = 0.005$, $n = 39$). Weak but significant positive correlation was found in Rothamsted ($r = 0.28$, $n = 50$, $p = 0.05$). Meanwhile, in Michigan ($r = 0.78$, $p < 0.001$, $n = 45$) and New Zealand ($r = 0.68$, $p < 0.001$, $n = 51$) the sensitivity for the pair of fungicides tended to increase together and had a strong coefficient of correlation (Figure 2.32).

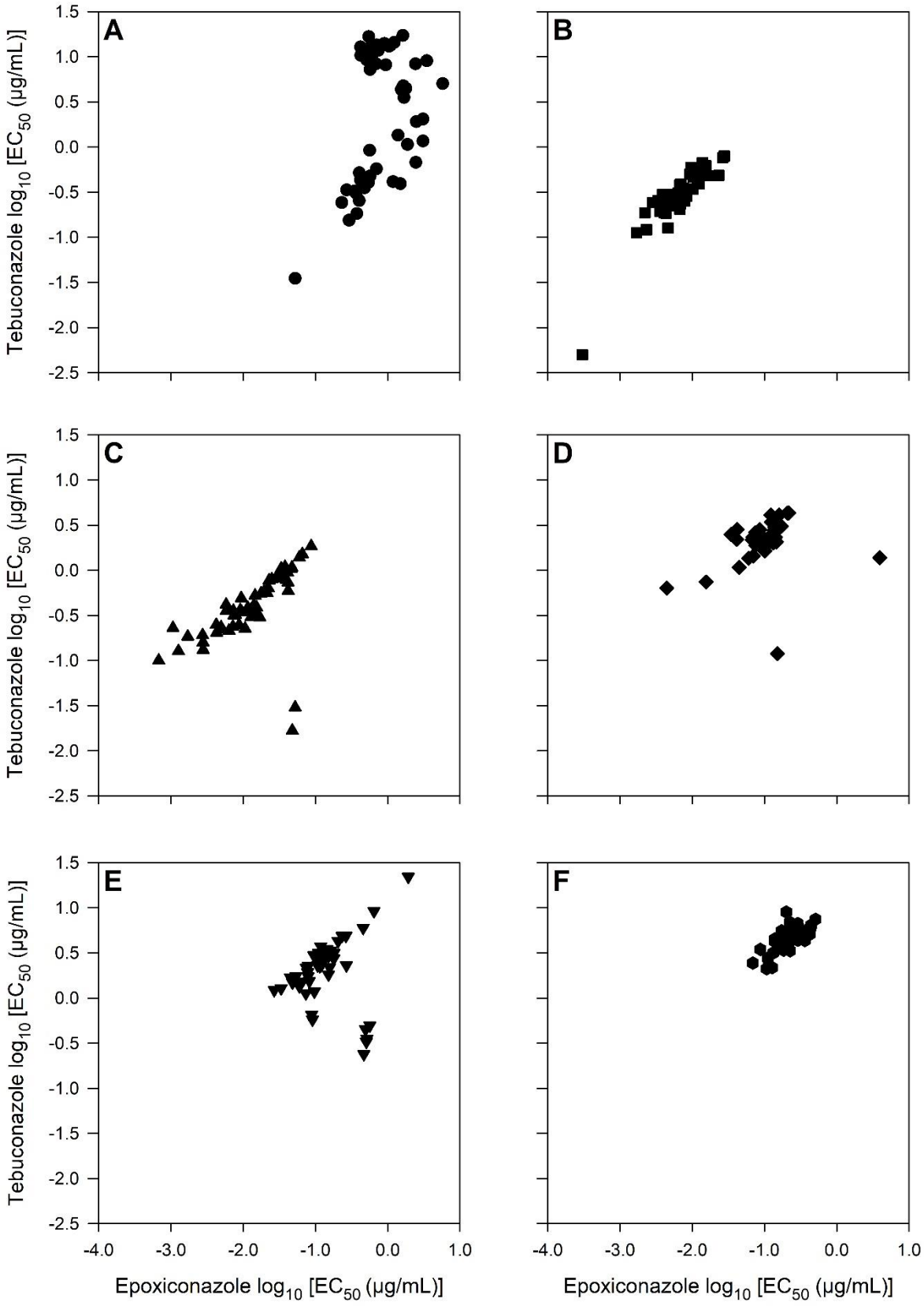

Figure 2.30 Correlation between epoxiconazole and tebuconazole log_{10} EC_{50} values of *Zymoseptoria tritici* field populations from Rothamsted-UK (A, n=50), Michigan-USA (B, n=45), Oregon-USA (C, n=57), Slovakia (D, n=39), Czech Republic (E, n=47) and New Zealand (F, n=51).

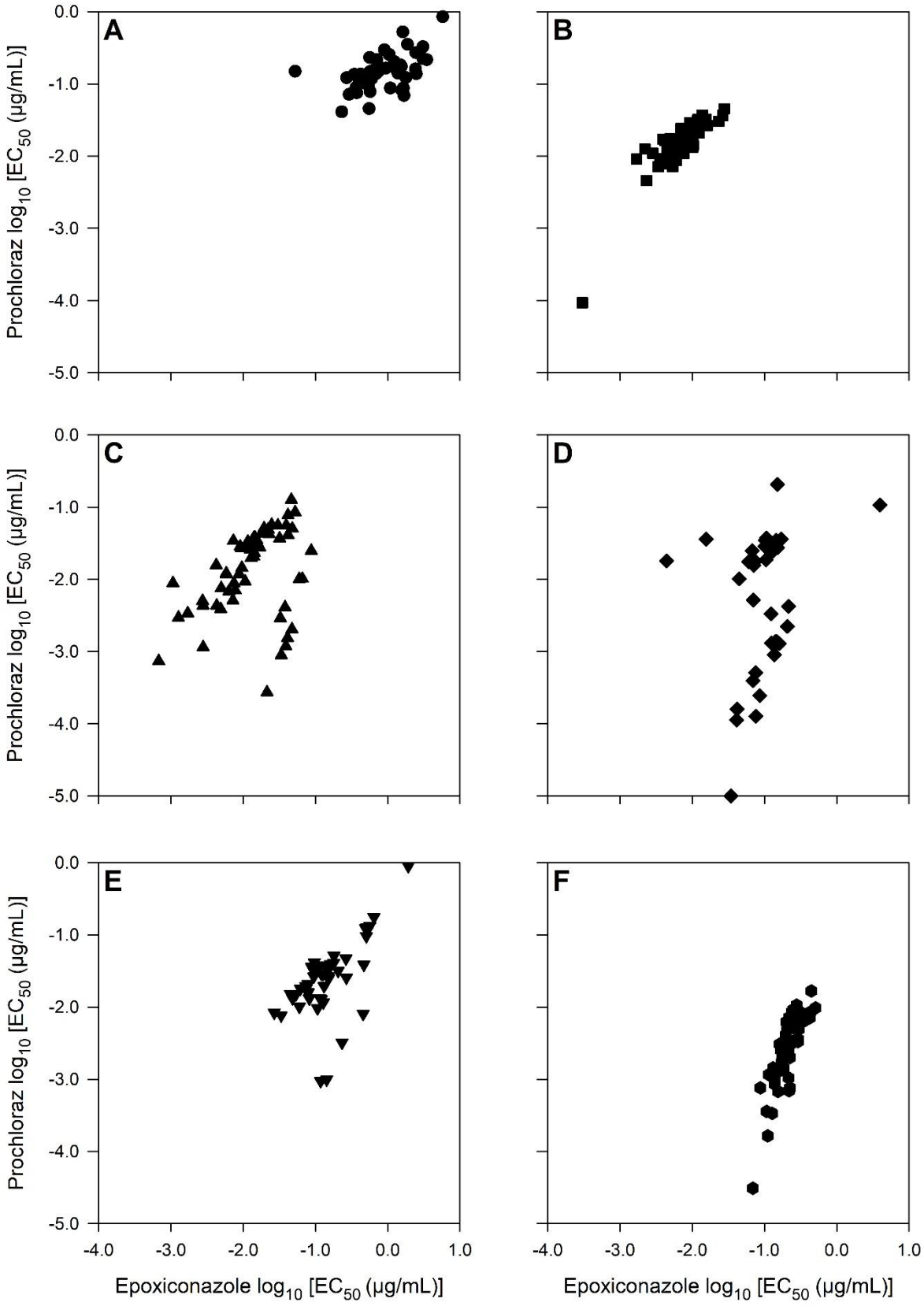

Figure 2.31 Correlation between epoxiconazole and prochloraz log_{10} EC_{50} values of *Zymoseptoria tritici* field populations from Rothamsted-UK (A, n=50), Michigan-USA (B, n=45), Oregon-USA (C, n=57), Slovakia (D, n=39), Czech Republic (E, n=47) and New Zealand (F, n=51).

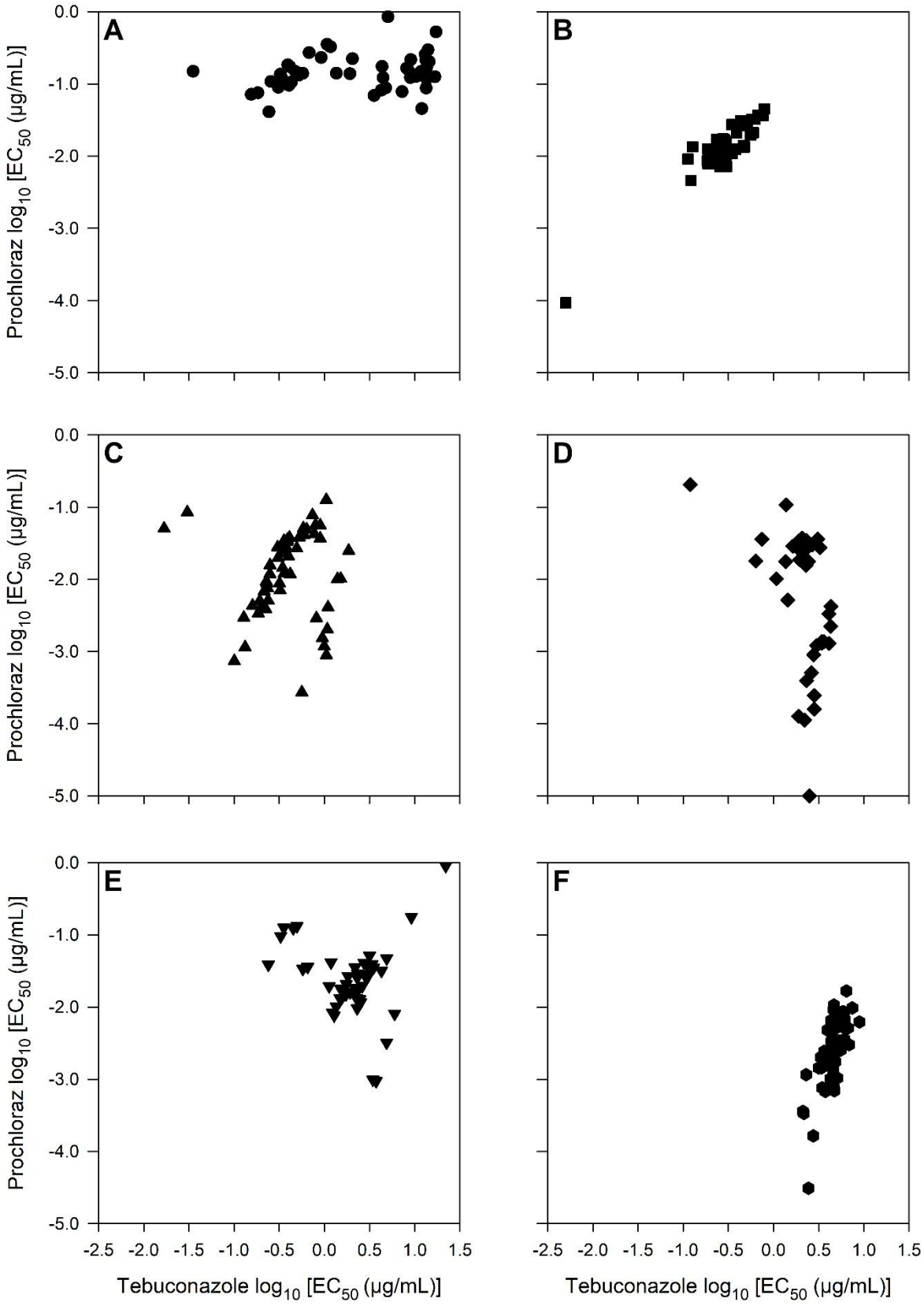

Figure 2.32 Correlation between tebuconazole and prochloraz log_{10} EC_{50} values of *Zymoseptoria tritici* field populations from Rothamsted-UK (A, n=50), Michigan-USA (B, n=45), Oregon-USA (C, n=57), Slovakia (D, n=39), Czech Republic (E, n=47) and New Zealand (F, n=51).

2.3.2.3 Azoxystrobin

Azoxystrobin sensitivity showed the characteristic high resistance factors associated with QoI resistance in all populations (Table 2.11).

Table 2.11 Range of QoI, multisite and SDHI fungicides resistance factors of *Zymoseptoria tritici* field populations from Rothamsted-UK, Chile, Argentina, Ruta 50-UY, La Estanzuela-UY, Michigan-USA, Oregon-USA, Slovakia, Czech Republic, and New Zealand.

Population	n	Azoxystrobin	Chlorothalonil	Bixafen	Fluopyram
		(Range of RF)[1]	(Range of RF)[1]	(Range of RF)[1]	(Range of RF)[1]
Rothamsted	50	60.5 - 814	0.23 - 2.68	0.23 - 9.88	0.19 - 43.4
Chile	71	0.12 - 338	0.23 - 2.67	0.22 - 6.60	0.03 - 5.90
Argentina	67	0.63 - 599	0.33 - 3.69	0.25 - 7.93	0.28 - 23.0
Ruta 50	53	0.64 - 663	0.48 - 5.44	0.08 - 10.5	0.31 - 12.4
La Estanzuela	42	0.24 - 879	0.20 - 5.26	0.13 - 14.4	0.52 - 16.8
Michigan	45	0.07 - 769	0.29 - 2.75	0.08 - 5.94	0.37 - 33.3
Oregon	57	0.39 - 769	0.36 - 3.09	0.32 - 13.9	0.59 - 154
Slovakia	39	0.47 - 508	0.18 - 4.36	0.54 - 10.9	0.87 - 32.4
Czech Republic	47	0.45 - 1021	0.28 - 5.37	0.18 - 13.2	0.51 - 124
New Zealand	51	0.51 - 469	0.54 - 2.93	0.29 - 2.76	0.31 - 20.7

[1]Resistance factor was calculated by dividing absolute values of the lowest EC_{50} and the highest EC_{50} in the range by IPO323 EC_{50}.

Isolates were grouped as sensitive [$\log_{10} EC_{50} < 0$ ($EC_{50} < 1$ ppm)] and resistant [$\log_{10} EC_{50} \geq 0$ ($EC_{50} \geq 1$ ppm)] to compare mean $\log_{10} EC_{50}$ values between groups. There was a significative difference of the mean $\log_{10} EC_{50}$ values between population groups among sensitive or resistant isolates ($p < 0.001$), nevertheless the mean between populations of the same group did not differ in any case (Table 2.12).

Table 2.12 ANOVA in the predicted means of azoxystrobin sensitive and resistant groups.

	Sensitive (EC_{50} < 1 µg/mL)		Resistant (EC_{50} ≥ 1 µg/mL)	
	Log_{10} EC_{50}±SE	P	Log_{10} EC_{50}±SE	P
Group	-	<0.001	-	<0.001
Rothamsted[1]	-	-	0.718 ± 0.032	-
South America[2]	-1.683 ± 0.026	0.184	0.831 ± 0.018	0.184
North America[3]	-1.574 ± 0.035	0.354	0.892 ± 0.032	0.354
Eastern Europe[4]	-1.491 ± 0.037	0.075	0.929 ± 0.035	0.075
New Zealand[5]	-1.602 ± 0.081	-	0.734 ± 0.036	-
LSD (5%)	0.1066	-	0.1066	-

[1]There are no sensitive isolates.
[2]South America: Chile, Argentina, Ruta 50 and La Estanzuela.
[3]North America: Michigan and Oregon.
[4]Eastern Europe: Slovakia and Czech Republic.
[5]2 isolates from New Zealand were excluded from the analyses

Generalized Linear Model (GLM) was applied to compare the proportions of sensitive and resistant isolates between groups and populations. The mean proportions for each group and population were used to calculate the predictions from the regression model, which are interpreted as the probability of resistance for each group or population (Table 2.13). Analyses of deviance detected differences in the probability of resistant isolates both between, groups and between populations ($p < 0.001$ in all cases).

Table 2.13 Predictions for azoxystrobin resistance from regression model.

Population	n	Azoxystrobin sensitivity		Predictions for Resistance		
		Sensitive (EC_{50} < 1 ppm)	Resistant (EC_{50} ≥ 1 ppm)	Prediction[1]	Lower CI[2]	Upper CI[2]
Rothamsted	50	0	50	0.999	0.929[3]	1[4]
Chile	71	57	14	0.197	0.120	0.306
Argentina	67	5	62	0.925	0.833	0.969
Ruta 50	53	4	49	0.925	0.815	0.972
La Estanzuela	42	13	29	0.691	0.537	0.811
Michigan	45	32	13	0.289	0.175	0.437
Oregon	57	18	39	0.684	0.553	0.791
Slovakia	39	35	4	0.103	0.039	0.244
Czech Republic	47	8	39	0.830	0.695	0.913
New Zealand[5]	49	8	41	0.837	0.706	0.916

[1]Predictions were calculated on logit scale and back-transformed for population means.
[2]95% confidence intervals were calculated on logit scale and back-transformed.
[3]Rothamsted lower limit was calculated using the Clopper-Pearson binomial interval.
[4]Rothamsted upper confidence limit was fixed at 1.
[5]2 isolates from New Zealand were excluded from the analyses.

Differences between populations can be compared using predictions on logit scale. All isolates in Rothamsted were resistant to azoxystrobin and because there is no variation in the population the Least Significative Difference (L.S.D) used to compare it to other populations is not meaningful. Regarding other comparisons, Chile had smaller probability for resistance than the other South American samples. Michigan and Slovakia had lower probability for resistance than Oregon and Czech Republic, respectively. Argentina, Ruta 50, Czech Republic and New Zealand had the highest values for probability of resistance other than Rothamsted (Figure 2.33).

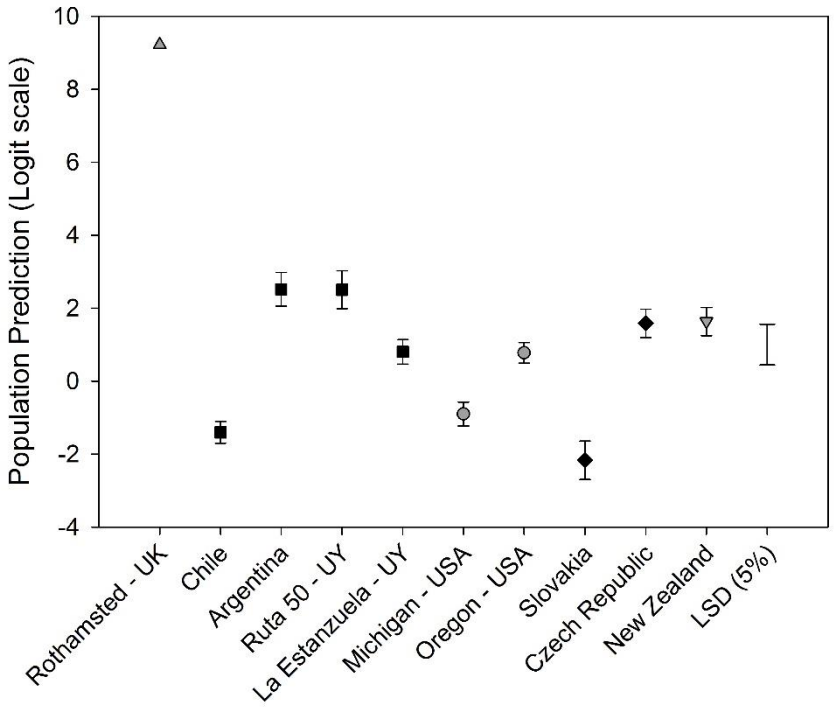

Figure 2.33 Prediction for azoxystrobin resistance of *Zymoseptoria tritici* field populations from Rothamsted-UK (n=50), Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53), La Estanzuela-Uruguay (n=42), Michigan-USA (n=45), Oregon-USA (n=57), Slovakia (n=39), Czech Republic (n=47) and New Zealand (n=51). Bars represent the standard error.

2.3.2.4 Chlorothalonil

Chlorothalonil sensitivity was different between groups ($p = 0.001$), additionally, the mean $\log_{10} EC_{50}$ values were higher for all other groups than for Rothamsted. Regarding populations within the same group, there was significative difference between South American samples ($p < 0.001$) (Table 2.14).

Even though the ANOVA detected significant differences, the mean $\log_{10} EC_{50}$ values did not vary more than 1.38-fold between samples (Rothamsted – Ruta 50) (Figure 2.34). Furthermore, resistance factors ranged similarly between populations and larger shifts in sensitivity were not observed (Table 2.11).

Table 2.14 ANOVA in the predicted means of chlorothalonil, bixafen and fluopyram sensitivities for groups.

	Chlorothalonil		Bixafen		Fluopyram	
	Log_{10} EC_{50}±SE	P	Log_{10} EC_{50}±SE	P	Log_{10} EC_{50}±SE	P
Group	-	0.001	-	<0.001	-	<0.001
Rothamsted	-0.941±0.037	-	-1.188±0.054	-	-0.551±0.064	-
South America[1]	-0.783±0.017	<0.001	-1.254±0.025	0.006	-0.837±0.029	<0.001
North America[2]	-0.838±0.026	0.288	-1.052±0.037	0.505	-0.136±0.045	<0.001
Eastern Europe[3]	-0.787±0.028	0.065	-1.105±0.041	0.017	-0.445±0.049	0.640
New Zealand	-0.778±0.036	-	-1.418±0.053	-	-0.859±0.064	-
LSD (5%)	0.081	-	0.119	-	0.143	-

[1]South America: Chile, Argentina, Ruta 50 and La Estanzuela.
[2]North America: Michigan and Oregon.
[3]Eastern Europe: Slovakia and Czech Republic.

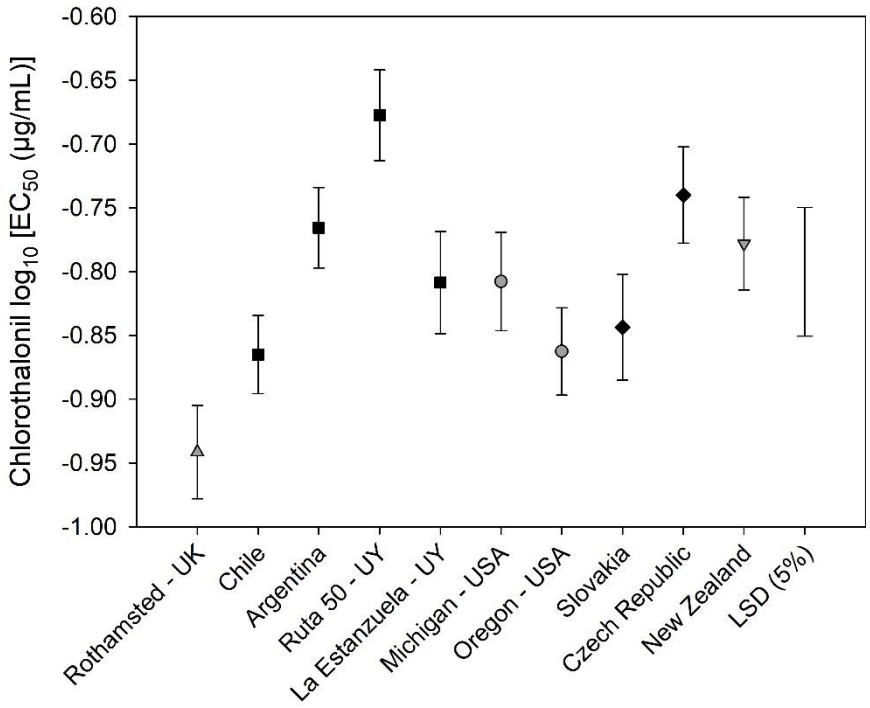

Figure 2.34 Mean of chlorothalonil log_{10} EC_{50} values of *Zymoseptoria tritici* field populations from Rothamsted-UK (n=50), Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53), La Estanzuela-Uruguay (n=42), Michigan-USA (n=45), Oregon-USA (n=57), Slovakia (n=39), Czech Republic (n=47) and New Zealand (n=51). Bars represent the standard error.

2.3.2.5 SDHI fungicides

The combined ANOVA for bixafen and fluopyram showed that sensitivity behaved differently across the groups for the two fungicides ($p < 0.001$), therefore they were analysed separately. Bixafen and fluopyram sensitivity varied between the groups ($p < 0.001$). Likewise, the mean log_{10} EC_{50} of both fungicides showed significant differences between populations within groups, except for bixafen in North America ($p = 0.505$) and fluopyram in Eastern Europe ($p = 0.06$) (Table 2.14).

Even though there were significative differences between population means for bixafen (Figure 2.35) it is noticeable that resistance factors were no larger than 14.4 folds compared to the wild type (Table 2.11). In contrast, the mean of fluopyram log_{10} EC_{50} in Oregon was considerable higher than any other population (Figure 2.36). Also, the range of resistance factors for fluopyram showed the presence of insensitive isolates in all populations but Chile (Table 2.11).

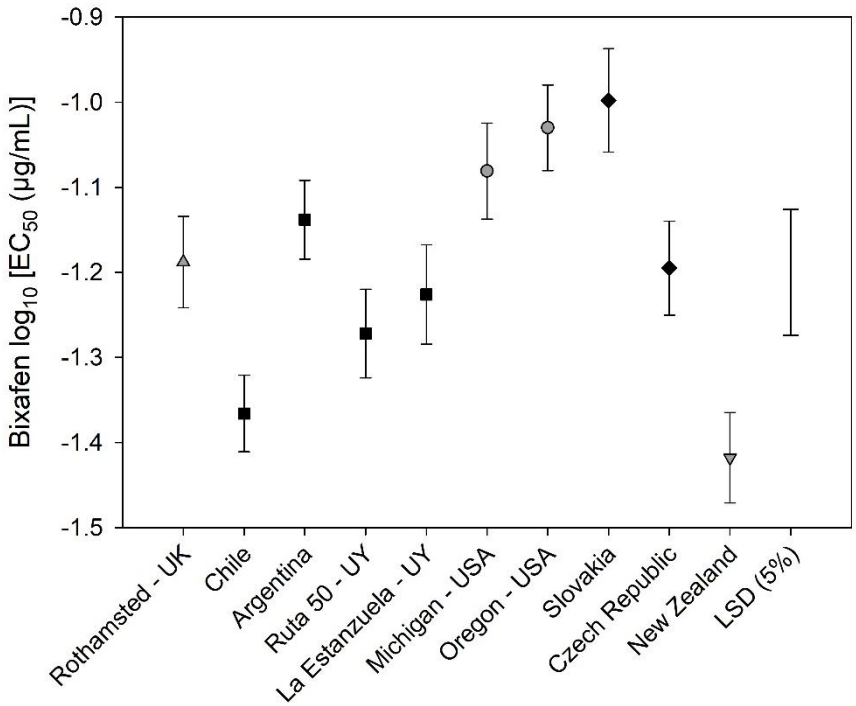

Figure 2.35 Mean of bixafen log_{10} EC_{50} values of *Zymoseptoria tritici* field populations from Rothamsted-UK (n=50), Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53), La Estanzuela-Uruguay (n=42), Michigan-USA (n=45), Oregon-USA (n=57), Slovakia (n=39), Czech Republic (n=47) and New Zealand (n=51). Bars represent the standard error.

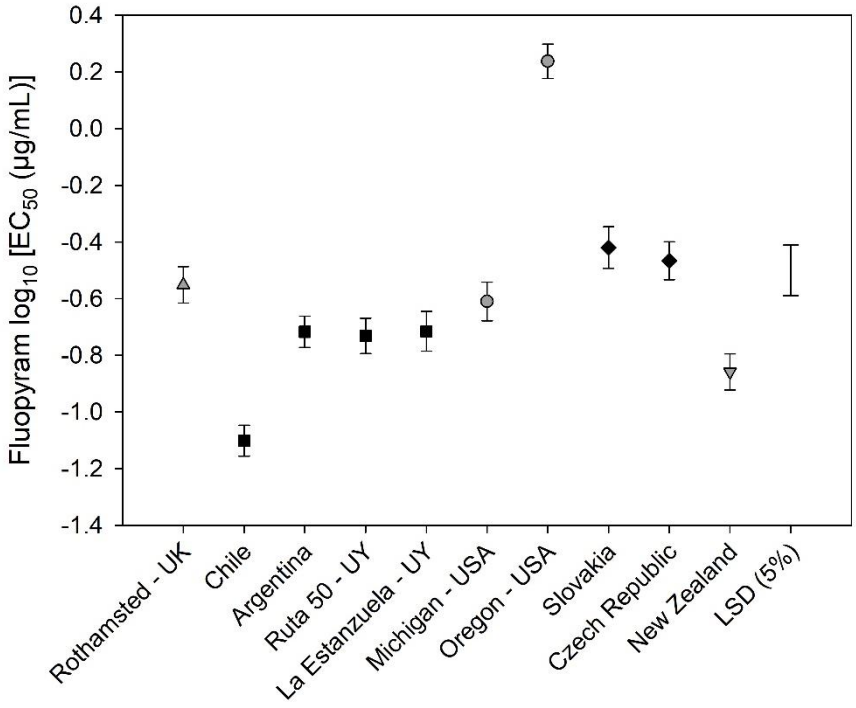

Figure 2.36 Mean of fluopyram log_{10} EC_{50} values of *Zymoseptoria tritici* field populations from Rothamsted-UK (n=50), Chile (n=71), Argentina (n=67), Ruta 50-Uruguay (n=53), La Estanzuela-Uruguay (n=42), Michigan-USA (n=45), Oregon-USA (n=57), Slovakia (n=39), Czech Republic (n=47) and New Zealand (n=51). Bars represent the standard error.

2.3.2.6 Cross-resistance of SDHI fungicides

Spearman's correlation coefficients were calculated for log_{10} EC_{50} values to quantify cross-resistance among bixafen and fluopyram. There was strong correlation between the pair of fungicides for all populations (Rothamsted, r = 0.56, n = 50; Michigan, r = 0.68, n = 45; Oregon; r = 0.69, n = 57; Slovakia, r = 0.63, n = 39; Czech Republic, r = 0.75, n = 47 and New Zealand, r = 0.65, n = 51, $p < 0.001$ in all cases), with except of a few individuals in each population with high insensitivity to fluopyram independent of bixafen sensitivity. A proportion of strains from Oregon had markedly higher log_{10} EC_{50} values to fluopyram (Figure 2.37).

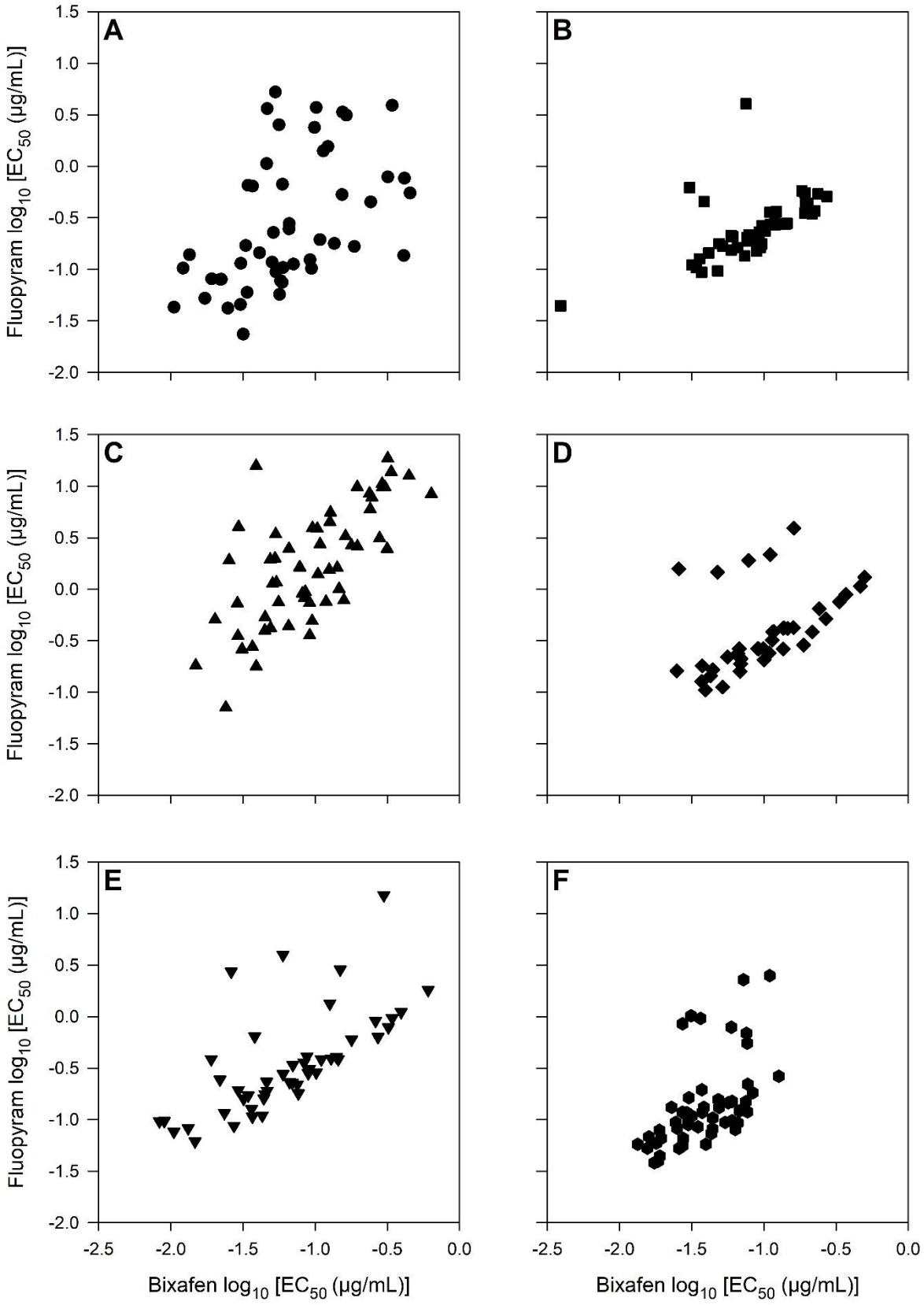

Figure 2.37 Correlation between bixafen and fluopyram log_{10} EC_{50} values of *Zymoseptoria tritici* field populations from Rothamsted-UK (A, n=50), Michigan-USA (B, n=45), Oregon-USA (C, n=57), Slovakia (D, n=39), Czech Republic (E, n=47) and New Zealand (F, n=51).

2.3.2.7 Multiple Drug Resistance and MBC resistance phenotypes

All 522 strains from the different continents had growth measured when exposed to 10 µg/mL of tolnaftate and 1.0 µg/mL of carbendazim to determine their MDR and MBC phenotypes, respectively. Only populations from Rothamsted, Oregon, and Czech Republic presented the MDR phenotype, with two strains each being able to grow on the concentration of 10 ppm of tolnaftate. Carbendazim resistance showed vast variation across populations (Table 2.15).

Table 2.15 Frequency of MDR and MBC resistance in *Zymoseptoria tritici field* populations from Rothamsted-UK, Chile, Argentina, Ruta 50-UY, La Estanzuela-UY, Michigan-USA, Oregon-USA, Slovakia, Czech Republic and New Zealand.

Population	n	Tolnaftate (10 µg/mL)[1]		Carbendazim (1.0 µg/mL)[1]	
		Sensitive strains	Resistance (%)	Sensitive strains	Resistance (%)
Rothamsted	49	47	4.0	3	94
Chile	71	71	0	18	75
Argentina	67	67	0	38	43
Ruta 50	53	53	0	12	77
La Estanzuela	42	42	0	31	26
Michigan	45	45	0	45	0
Oregon	57	55	3.5	44	23
Slovakia	39	39	0	10	74
Czech Republic	47	45	4.2	0	100
New Zealand	51	51	0	47	8

[1]Fungal growth was measured in two replicates for each isolate.

As per azoxystrobin, a Generalized Linear Model (GLM) was applied to compare the proportion of MBC resistant isolates between groups and populations. Analyses of deviance detected differences in the probability of carbendazim resistant isolates between groups and between populations belonging to the same group ($p < 0.001$ in all cases). The predictions from the regression model, interpreted as the

probability of resistance for each population, along with their confidence intervals, showed that none of the populations is similar to Michigan, because it is completely sensitive to carbendazim. In contrast, the population from Czech Republic was completely resistant to carbendazim and not different from Rothamsted (Table 2.16).

South American populations had variable behaviour for carbendazim resistance. While Chile and Ruta 50 had a high probability for resistance, similar to Slovakia, Argentina and Ruta 50 had lower predicted values, similar to Oregon. New Zealand was shown to have a low probability of resistance to carbendazim but was not completely sensitive as Michigan (Table 2.16).

Table 2.16 Predictions for carbendazim resistance from regression model.

Population	n	Predictions for carbendazim resistance				
		Logit Prediction	Logit LSD[1]	Prediction[2]	Lower CI[3]	Upper CI[3]
Rothamsted	49	2.730	1.065	0.939	0.826	0.980
Chile	71	1.080	1.065	0.746	0.633	0.834
Argentina	67	-0.270	1.065	0.433	0.319	0.553
Ruta 50	53	1.229	1.065	0.774	0.642	0.867
La Estanzuela	42	-1.036	1.065	0.262	0.151	0.414
Michigan[4]	45	-10.566	-	0	0	0.079
Oregon	57	-1.219	1.065	0.228	0.137	0.355
Slovakia	39	1.065	1.065	0.744	0.585	0.856
Czech Republic[4]	47	9.227	-	0.999	0.924	1
New Zealand	51	-2.464	1.065	0.078	0.029	0.191

[1]Average of all LSDs not involving Michigan and Czech Republic – LSDs for Michigan and Czech Republic cannot be calculated.
[2]Logit predictions were back-transformed for population means.
[3]95% confidence intervals were calculated on logit scale and back-transformed.
[4]Confidence intervals for Michigan and Czech Republic were calculated using Clopper-Pearson intervals.

2.4 Discussion

2.4.1 South American *Zymoseptoria tritici* field populations

Control of fungal diseases with extensive use of fungicides has become more common in wheat-growing regions all over the world. The southern cone of South America, the region that comprises Chile, Argentina, and Uruguay, has suitable conditions for the development of disease epidemics on wheat and as a result, fungicides have been extensively used by farmers to avoid yield losses (Kohli & Ackermann, 1998). Therefore, South American *Z. tritici* field populations have been under selection pressure from fungicide use since the 1990s (Andrade, 1990; Galich *et al.*, 1990; Ackermann, 2010). Due to the absence of *Z. tritici* isolates that have never been exposed to fungicides in South America, and the lack of a baseline sensitivity distribution prior to widespread fungicide use in that continent, we could only compare South American field populations to populations from other continents. All those populations have also been selected by chemical control to greater or lesser extents.

Azole sprays targeting Septoria tritici blotch in South America started in the late 1980s (Andrade, 1990; Galich *et al.*, 1990; Germán *et al.*, 1990) with the introduction of the first fungicides of this class. It then progressed throughout the 1990s with the launch of new azole molecules (Andrade, 1995; Ackermann, 1996; Rosa *et al.*, 2013). These are still in use but mostly in mixture with other fungicide classes, and even though the recent increase in fungicide usage was to target other wheat pathogens, *Z. tritici* populations were exposed to these applications (Jobet *et al.*, 2013; INIA - Uruguay, 2017; INTA - Argentina, 2018).

Azole field efficacy has remained stable in South American *Z. tritici* populations for several years but a decline in tebuconazole sensitivity in field isolates has been recently reported in Argentina (Cordo *et al.*, 2017). My study has detected the presence of isolates with loss of sensitivity to DMI fungicides in South American populations sampled in Chile, Argentina, and Uruguay. Shifts in azole sensitivity between the azole sensitive reference isolate IPO323 and modern field strains sampled in South America were as large as 1200-fold (in the case of epoxiconazole). The frequency distribution of

sensitivity to epoxiconazole, tebuconazole and prochloraz were left-skewed in most populations, showing that isolates have shifted to lower sensitivity over time. Data in this chapter provide evidence that azole resistance has been evolving in *Z. tritici* populations in South America. In addition, strong cross-resistance between the pair of fungicides epoxiconazole and tebuconazole was evident in all four population samples.

The azole sensitivity pattern found in this study was not the same in the four populations sampled from South America. In general, Chilean sensitivity was bimodally distributed, with some isolates being more insensitive than any other isolates from South America. The population sampled in Chile seems to be composed of subpopulations, and these had distinct patterns of cross-resistance between azoles. The selection pressure resulting from continuous fungicide exposure is proportional to dose (Mikaberidze *et al.*, 2017) and spraying frequency. Climate conditions where this population from Chile was sampled tend to be more favourable for *Z. tritici* than those in Argentina and Uruguay, leading to higher disease pressure and consequently more fungicide usage, resulting in higher selection for less sensitive strains (Caglevic, 1982; Andrade & Contreras, 2007). Moreover, Chile is isolated from other countries in South America by the Andes and consequently may have evolved fungicide resistance differently from populations located in Argentina and Uruguay. These are located eastwards of the Andes and all lay within a circle of 180 km, where gene flow can be substantial between regions due to ascospores movements in a contiguous area sown with wheat.

The sexual stage of *Z. tritici*, *Mycosphaerella graminicola*, was proved to play a major role in the pathogen dispersal in South America (Banke & McDonald, 2005; Castillo *et al.*, 2010; Simón *et al.*, 2012), then each sample from a single location was expected to be composed of isolates representative of a substantially broader region. Although the largest differences in azole sensitivities were found between Chile and the other populations, there were also significant differences in sensitivities between Ruta 50 and La Estanzuela, sampled only six km apart in Uruguay. These variations between field populations probably resulted from different usage of azoles at field scale in previous seasons,

leading to a site-specific selection of strains that would serve as inoculum next season. Even though azole fungicides target the same enzyme, different chemical molecules in this class bind in a very specific manner to the target site, and consequently select different CYP51 variants (Fraaije *et al.*, 2007). Fungicide usage in the previous season can drive locally different patterns of fungicide resistance because ascospores from local wheat debris are dominant inoculum sources (Suffert *et al.*, 2011; Garnault *et al.*, 2019).

Just as with azoles, the results of this study show that resistance for QoI and MBC fungicides is widespread in South American populations of *Z. tritici*. This was expected as extensive fungicide use has led to rapid selection for resistance to these fungicide classes in Europe (Fraaije *et al.*, 2003, 2005; Lucas *et al.*, 2015). Different spatial patterns of *Z. tritici* resistance were observed for azoxystrobin (QoI) and carbendazim (MBC) fungicides. In South America all four populations showed a typical bimodal shape for azoxystrobin cumulative frequency distributions, comprising sensitive and resistant subpopulations. However, the frequency of resistant isolates was lower in Chile than in Uruguay (Ruta 50 and La Estanzuela) and Argentina. This corroborates the results for azole sensitivity, which showed a different pattern of resistance in Chile compared to other samples from South America. In contrast, Chile had a similar frequency of MBC resistance to Ruta 50, which was significantly higher than in the adjacent samples, Argentina and La Estanzuela.

2.4.2 Global *Zymoseptoria tritici* field populations

Moving to the global scale, differences in azole sensitivity were observed in all samples that were tested in this study. Incomplete cross-resistance between different members of the azole class was also detected in all samples. The two North American samples were more sensitive than all other populations analysed in this study, except to prochloraz. Use of fungicides in North American wheat production is more recent and less intense than in Europe, though in the Willamette Valley of Oregon wheat producers have increased from 1 or 2 fungicide applications per year during the 1990s to 3 to 4 applications of azoles in mixture with strobilurins after severe epidemics of stripe rust (*Puccinia*

striiformis) in mid-2000s (Estep *et al.*, 2015). More recently, mixtures of the azoles prothioconazole and tebuconazole have been introduced in the Willamette Valley/Oregon, although propiconazole and tebuconazole are by far the most applied fungicides in this region (Sykes *et al.*, 2018). The population from Oregon used in this study was sampled in an area not exposed to intense fungicide usage and relatively isolated from the commercial wheat production region of Oregon. Nevertheless, some strains from both North American populations had azole sensitivity less than the azole sensitive reference isolate sampled in Europe a few decades ago. In Oregon, decreased sensitivity to propiconazole has been previously reported in Willamette Valley along with loss of field effectiveness (Hayes *et al.*, 2016; Hagerty *et al.*, 2017), while slight azole cross-resistance was detected in Oregon isolates sampled in 2012 (Sykes *et al.*, 2018). Fungicide resistance has not been reported for *Z. tritici* in other areas of North America. Gene flow between North American *Z. tritici* populations from Oregon and Michigan was not expected since they were sampled more than 3,000 km apart and there was no contiguous area sown with wheat between them. However, azole sensitivity was rather similar between these two populations. Similar azole fungicide usage between these locations may have led to parallel selection for less sensitive strains. In contrast, different patterns of azoxystrobin and carbendazim resistance suggest that the pattern of QoI and MBC use must have been different between regions. Thus, resistance seems to reflect fungicide use closely in independent populations. MBC resistance in *Z. tritici* has been present in the Willamette Valley region since the 1980s (Hagerty *et al.*, 2017). Rothamsted has not been exposed to MBC selection in the past few decades and still has a fixed MBC resistance of 94% in frequency, evidencing stability and lack of fitness cost associated with this type of resistance. Allied to the quick development of MBC resistance, the introduction of modern azole fungicides and later their mixtures with strobilurins probably caused the use of MBC compounds on wheat in Oregon to cease, comparable to what happened in Europe (Lucas & Fraaije, 2008; Lucas *et al.*, 2015).

Strobilurins have been used for STB control since 2004 in Oregon (Hagerty *et al.*, 2017), and QoI resistance was first detected in 2012 (Estep *et al.*, 2013). The finding in the present study of 68% azoxystrobin resistant phenotype in Oregon is lower than the 93% resistant phenotype found by

Hagerty *et al.* (2017) in a broad spatial-temporal survey in the Willamette Valley between 2014-2016. The same authors reported only 22% azoxystrobin resistant phenotype in 2015 at the same site the population in this study was sampled. Thus my results are consistent with the hypothesis that long-distance dispersal of ascospores may be spreading resistant alleles in Western Oregon, as suggested by Hagerty *et al.* (2017). The same selection and spread of resistance are to be expected in Michigan, where only 29% of azoxystrobin resistance phenotype was detected in the population sampled in 2018.

In vitro sensitivity of *Z. tritici* field populations sampled at Rothamsted-UK has decreased by 17 and 20 fold between 2003 and 2015 to epoxiconazole and prothioconazole-desthio, respectively (Blake *et al.*, 2018). Although in this study the mean EC_{50} values at field populations from Slovakia and Czech Republic were lower to epoxiconazole and prochloraz compared to that from Rothamsted, some isolates sampled in these two populations had azole sensitivity profiles comparable with the most resistant isolates from Rothamsted. STB has gained importance in Czech Republic since the early 2000s, causing damage to wheat production in 2002 and 2003 (Tvaružek *et al.*, 2012). It has been proposed that mutations conferring low azole sensitivity in *Z. tritici* arise in NW-European countries, were fungicide selection pressure is intense and has a well-known history, then migrate eastwards into wheat-growing countries through long-distance dispersal of ascospores along with wind currents (Brunner *et al.*, 2008). Differences between the overall population mean sensitivities across the European continent are presumed to be due to the distribution of azole-resistant isolates, at higher frequencies in NW-populations where high levels of azole fungicides are used. Lower fungicide spraying and consequently low selection pressure in wheat fields in Eastern Europe, perhaps associated with fitness costs in highly resistant strains, keep azole resistance at a low level.

The same assumption of ascospores movement causing resistant allele dispersal west to east can be assumed for carbendazim because both Eastern European sites had high resistance frequencies. In contrast, the frequency of azoxystrobin resistant strains was much higher in Czech Republic (83%) than Slovakia (10%). Tvaružek *et al.* (2012) have found signs of eroding sensitivity to azoxystrobin working

with isolates from various locations within the Czech Republic sampled in 2002. Nonetheless, resistant alleles at the *cyt b* locus were only found in 2007, then they almost completely replaced sensitive alleles within six years following increased use of strobilurins in the Czech Republic (Drabešová *et al.*, 2013). It is already known that QoI resistant strains arose independently in different genetic backgrounds (Torriani *et al.*, 2009) and airborne ascospores potentially disperse resistant alleles over many kilometres (Fraaije *et al.*, 2005). These differences between frequency in Czech Republic and Slovakia could mean resistance is present but the strength of selection and therefore rate of frequency increase may vary in Eastern European populations. Alternatively, the difference could be due to the higher gene flow of resistant alleles in the direction of the Czech Republic. Drabešová *et al.* (2013) has assessed gene flow among different regions within the Czech Republic and found a higher influx of migrants into Kroměříž, the same location in which the population in this study was sampled. These authors proposed that the reason for higher gene flow in direction of Kroměříž was due the presence of an important wheat breeding station in the region and could be associated with the movement of infected plant material to this station. The independent emergence of QoI resistant alleles within the Czech Republic could also be the reason for the higher frequency of QoI resistant strains in comparison to Slovakia.

STB has regained importance in New Zealand in the late 1990s, at which time mixtures of DMIs and QoIs were used up to three times in a wheat season to avoid yield losses (Cromey *et al.*, 2000; Marroni *et al.*, 2006). More recently, two to four fungicide applications, including SDHIs, have been used to control not only STB but also other important fungal diseases in autumn-sown wheat (Stewart *et al.*, 2014). In this research, we found that a *Z. tritici* field population sampled in the southern North Island region in New Zealand had a loss of sensitivity to epoxiconazole and tebuconazole, but was very sensitive to prochloraz. In addition, azoxystrobin resistance was present in 83% of the isolates sampled. These data corroborate findings from Stewart *et al.* (2014) when the authors found azoxystrobin resistance to be widespread and epoxiconazole sensitivity shifted towards resistance. The last two wheat-growing seasons (2016-17) before the population in the present study was sampled were wetter than in previous years (2014-15), consequently higher STB pressure was observed (FAR, 2018). With

the widespread evolution of QoI resistance (Stewart *et al.*, 2014) SDHIs have been more often applied in mixtures with azoles (FAR, 2018). Even with the increased use of this fungicide class in the last seasons, this research has not yet found signs of SDHI resistance in New Zealand. Interestingly, as in Michigan, most of the New Zealand population was sensitive to the MBC carbendazim. MBC use on wheat was very low in New Zealand a decade ago (van Toor *et al.*, 2013). My hypothesis is that this class of fungicide has not been extensively used in these two regions because there was no demand for it with the introduction of more modern fungicides in the market (i.e.: azoles and strobilurins). Thus, they could be used now in a spraying program of management of fungicide resistance in mixture with other compounds.

2.4.3 Multisite inhibitor and SDHI fungicides

Resistance tends to emerge quickly where growers cultivate susceptible cultivars and rely extensively on single-site fungicides. Mavroeidi & Shaw (2006) described that the greatest selection for resistance occurred with the best treatment for disease control, in the case when the azole fluquinconazole was applied as a solo product. Depending on the genetic basis of resistance and the fitness costs associated with the resistance in the presence or absence of the fungicide, resistance may occur immediately after fungicide introduction or after many years. Resistance to QoI fungicides arises rapidly and completely so fungicide effectiveness is definitively lost. In the case of azole fungicides, resistance evolves slowly and progressively with the increasing number of point mutations in the target site linked to loss of fungicide sensitivity, as it has been seen in NW-European populations of *Z. tritici* over the years (Blake *et al.*, 2018). Despite substantial differences among samples, in this study, we demonstrated that all *Z. tritici* field populations have reduced sensitivity to azoles. We have also shown that QoI resistance is widespread in populations from different continents where *Z. tritici* has some importance as a threat to wheat production. Blake *et al.* (2018) have demonstrated that increased *in vitro* EC_{50} values are correlated with an increased field ED_{50}. Therefore, the effectiveness of fungicide control is compromised due to the emergence of strains with reduced sensitivity that can outcompete native isolates in an environment of fungicide selection pressure.

As it has been shown, populations of *Z. tritici* vary in sensitivity to different azole molecules, hence good field performance in STB control can often be obtained with some active ingredients from this class. Careful spray timing to maximise preventative rather than curative activity can also lead to more effective control where some sensitivity shifts have occurred. These products can be mixed with SDHIs and chlorothalonil to ensure good efficacy in disease control and retard azole resistance evolution (Hobbelen *et al.*, 2014; Heick *et al.*, 2017). The multisite inhibitor fungicide chlorothalonil did not show a major sensitivity shift in any strain sampled, proving its value worldwide as a mixing partner to DMIs and SDHIs in an anti-resistance strategy. In the SDHI class, the spectrum of sensitivity to bixafen was within the sensitive range in all populations, whereas fluopyram showed low sensitivity in a selection of isolates distributed in all populations, except Chile. These were highly resistant to fluopyram but not cross-resistant to bixafen. Oregon had a much lower sensitivity to fluopyram because of a higher frequency of individuals with high resistance factors. Nevertheless, most populations in this study were sampled in 2016 and since then isolates of *Z. tritici* carrying target site mutations with low to high resistance factors towards SDHI fungicides have been found in Europe (FRAG-UK, 2015; FRAC, 2016; Dooley *et al.*, 2016a; Rehfus *et al.*, 2018).

Chapter 3: Molecular mechanisms of fungicide resistance in *Zymoseptoria tritici*

3.1 Introduction

The previous chapter demonstrated that evolutionary changes in response to the use of fungicide have occurred and are occurring in South American and other populations of *Zymoseptoria tritici*. This raises the question of the mechanisms underlying these changes.

Methyl Benzimidazole Carbamates (MBCs) were the first systemic fungicides to be used in STB control, worldwide (Andrade, 1990; Galich *et al.*, 1990; Germán *et al.*, 1990; Kema, 1990; Hagerty, 2016). Resistance to MBCs in *Z. tritici* was widespread by the mid-1980s across Europe and in the USA (Lucas *et al.*, 2015; Hagerty, 2016). This was rapidly shown in *Z. tritici,* as in many other fungi, to arise from a single base mutation in the β-tubulin encoding gene, leading to the emergence of the E198A variant allele. This substitution causes high levels of resistance across all classes of MBC fungicides and has no apparent fitness costs in *Z. tritici*. Therefore, E198A alleles persist in high frequency in NW-European populations of *Z. tritici*, even though the use of these fungicides has long been discontinued in wheat (Lucas *et al.*, 2015; Young, 2015).

Introduced in the late 1990s, the Quinone outside Inhibitors (QoIs) initially showed excellent activity against STB inhibiting mitochondrial respiration (Sierotzki, 2015). However, as for MBCs, resistance emerged quickly due to point mutations in the target site encoding gene *cytochrome b*, particularly that leading to amino acid alteration G143A (Fraaije *et al.*, 2003) which increased resistance by factors of thousands in lab assays. Due to the fixation of the G143A allele in Europe (Torriani *et al.*, 2009), and other continents (Estep *et al.*, 2013; Stewart *et al.*, 2014), QoI fungicides became drastically less effective in STB control.

Azole fungicides, also known as Demethylation inhibitors (DMIs), have been the backbone of STB control in many countries where this disease reduces wheat yield. Compounds within this class bind to the P450-sterol 14α-demethylase enzyme (CYP51) and inhibit fungal growth due to the resulting ergosterol depletion (Ziogas & Malandrakis, 2015). Shifts in azole sensitivity have been identified since

the mid-1990s in the UK (Mavroeidi & Shaw, 2005; Brunner *et al.*, 2008; Lucas *et al.*, 2015). Unlike with MBC and QoI fungicides, the various *CYP51* mutations in *Z. tritici* do not confer full cross-resistance to the whole class and resistance has developed gradually with a stepwise accumulation of amino acid changes in CYP51, earlier appearing mutations facilitating the emergence of later ones conferring greater resistance (Leroux *et al.*, 2007; Cools *et al.*, 2011; Mullins *et al.*, 2011; Hawkins *et al.*, 2019). The azole sensitivity shifts in *Z. tritici* also involve other resistance mechanisms, such as overexpression of the *CYP51* gene, non-target site efflux fungicide by MFS1 transmembrane efflux transporters and melanization (Cools *et al.*, 2012; Lendenmann *et al.*, 2015; Omrane *et al.*, 2015). Modern *Z. tritici* European populations have a wide range of sensitivity because the varied use of different azoles tends to select specific CYP51 variants (Dooley *et al.*, 2016b; Blake *et al.*, 2018). Even though sensitivity to DMIs has declined in most European and some non-European populations, several azole compounds still provide good disease control and are frequently used in all wheat-growing regions.

The most recent fungicides introduced into the market for STB control belong to the new generation of Succinate Dehydrogenase Inhibitors (SDHIs). These fungicides are single-site inhibitors that target the succinate dehydrogenase in the respiration chain, an enzyme composed of four subunits, SdhA, SdhB, SdhC, and SdhD, of which three interact with SDHIs (Stammler *et al.*, 2015). Due to their high efficacy, SDHIs were quickly adopted by growers, applied either in alternation or mixture with DMIS. In 2012 the first field isolates with slightly shifted sensitivity to SDHIs were found in France and the UK, carrying amino acid substitutions C-T79N and C-W80S, respectively (Lucas *et al.*, 2015). In recent years, several target-site mutations conferring reduced SDHI sensitivity were identified across Europe, conferring low (i.e.: B-T268I and C-N86S) to moderate (C-H152R) resistance factors, although the overall frequency of C-H152R remains at a low frequency (Dooley *et al.*, 2016a; Kirikyali *et al.*, 2017; Rehfus *et al.*, 2018; FRAC, 2019). Isolates with enhanced efflux pump activity have reduced DMI and SDHI sensitivity (Omrane *et al.*, 2017). More recently a paralog of SdhC found in some strains of *Z. tritici* was reported to be a mechanism of resistance to a subclass of SDHIs that includes fluopyram (Steinhauer *et al.*, 2019).

In the previous chapter, South American field populations of *Z. tritici* showed reduced sensitivity to MBC, QoI, and azole fungicides. Moreover, a few isolates were demonstrated to have shifted sensitivity to fluopyram. The present chapter tests the hypothesis that South American field isolates of *Z. tritici* have evolved the same mechanisms of fungicide resistance first found in Europe. This was done by sequencing the encoding genes of the fungicide target sites. Additionally, with the sequence of the whole *CYP51* gene from a selection of isolates, it was possible to elucidate the global evolutionary history of CYP51 amino acid changes in *Z. tritici*.

3.2 Material and Methods

3.2.1 Origin of isolates

The *Z. tritici* isolates used in this study are a selection of the collections from Chapter 2, in which EC_{50} values of each strain were determined for different fungicide classes. Isolates with a range of fungicide resistance phenotypes were chosen to have the encoding gene of the respective target site sequenced without prior knowledge of their genotypes. To sequence the CYP51 encoding gene, 10 isolates per population were selected in a way that they represent the whole spectrum of azole sensitivities in each population. Twenty-three isolates with phenotypes of sensitivity and resistance to azoxystrobin and carbendazim were selected from all populations to sequence the encoding genes of the respective target sites. Additionally, 29 isolates with the highest EC_{50} values of bixafen and fluopyram were selected from all populations to have the encoding gene of SDH subunits sequenced.

3.2.2 DNA extraction

To extract the fungal DNA, the selected isolates were cultured in a petri dish with YPD agar and grown for five days at 15°C in the dark. DNA was extracted using the MasterPure™ Yeast DNA Purification Kit - MPY80200 (Lucigen, Middleton, WI, USA). Yeast like spores from five old day culture were scraped off from the petri dish with a 1 µL sterile loop, placed into a 0.5 mL tube filled with 100 µL of Yeast Cell Lysis Solution and 0.5 µL of RNase A and vortexed twice for 10 seconds. The samples were incubated in a water bath at 65°C for 15 minutes. Each sample was vortexed twice for 10 seconds and incubated

on ice for 5 minutes. In the next step, 50 µL of MPC Protein Precipitation Reagent was added per sample, then vortexed for 10 seconds, and then centrifuged at 13200 rpm for 10 minutes. The supernatant from the previous step was transferred to a clean tube and 200 µL of isopropanol added, then centrifuged at 13200 rpm for 10 minutes. The supernatant was discarded, the pellet washed with 100 µL of 70% ethanol and centrifuged for 5 minutes at 13200 rpm. The ethanol was discarded, and the DNA pellet air-dried and re-suspended in 200 µL of TE Buffer. DNA was quantified with a Nanodrop spectrophotometer (Nano Technologies, Delaware, USA), and further diluted to 20 ng/µL before storage at -20°C.

3.2.3 Polymerase chain reactions

Polymerase Chain Reaction (PCR) was carried out in a Biometra T3000/T3 thermocycler (Biometra GmbH, Göttingen, Germany). Primers sequences and reaction conditions for amplification of *CYP51*, *cytochrome b*, *β-tubulin*, and *Sdh* genes, as well as CYP51 and *Mg*MFS1 promoter inserts, are described in Table 3.1. Amplifications were performed in a 40 µL reaction volume containing 80 ng of template DNA, 0.5 µM each of forward and reverse primers, 4 µL of 10x Easy-A reaction buffer (Agilent Technologies, USA), 0.2 mM of each dNTP, and 2 U of Easy-A high-fidelity PCR cloning enzyme (Agilent Technologies). The only exception was for the PCR reaction to amplify the CYP51 promoter insert. This reaction was performed in a 20 µL reaction volume containing 40 ng of template DNA, 0.5 µM of each forward and reverse primers, 2 µL of 10x reaction buffer IV (Thermo Fisher Scientific, Loughborough, UK), 0.15 mM of each dNTP, and 0.2 U of Red Hot DNA Polymerase (Thermo Fisher Scientific).

The PCR products were mixed with a 20% (v/v) DNA loading dye (New England BioLabs, UK) and separated in 1.5% agarose gel stained with Ethidium Bromide. The gel electrophoresis ran at 100V for a variable time depending on the expected product size, in a tank with a 1xTBE buffer (0.8 M Tris Borate, 20 mM Na_2EDTA). After that, the gel was exposed to UV light at 320 nm in a transilluminator (Syngene, Maryland, USA) to visualize the DNA fragments. PCR products were sequenced using the two PCR primers, except for *CYP51*, where three additional primers (F_2, REV_901 and FOR_1100) were used to

cover the whole gene. PCR products were purified and sequenced by Eurofins Genomics (Ebersberg, Germany).

Table 3.1 Primer sequences and thermocycling conditions for amplification of fungicide target sites and promoter inserts.

Primer and sequences (5'-3')	Target amplification	Thermocycling conditions
FOR_01: GATTTATCGAACGATAATTTGGT[7] REV_01: TAGCATAAGATCCACCTATGGT[7] F_2: AGAAGTTCGCATCGAC[1,7] REV_901: CCTCCTGTGCCTGACTTC[1,7] FOR_1100: TGGAGACGATGCTGA[1,7]	CYP51	95°C – 2 min 40 cycles at 95°C – 10 s 57°C – 20 s 72°C – 2 min 72°C – 8 min
BTub_F: TGCCGTGCTCGGTCGATTTGGA[6] BTub_R: GGAGCGAAACCGACCATGAAG[6]	β-tubulin	95°C – 2 min 40 cycles at 95°C – 40 s 60°C – 30 s 72°C – 1 min 72°C – 7 min
Ztctb_F1: TACCTGACTGGTATCATATTGTGT[6] Ztctb_R1: TCCGTAAGATGGTATCTCTAAAGT[6]	Cytochrome b	95°C – 2 min 40 cycles at 95°C – 10 s 58°C – 20 s 72°C – 2 min 72°C – 8 min
SdhB_F: TAAACACTCCACGCCTCACG[2] SdhB_R: GTCTTCCGTCGATTTCGAGAC[6]	SdhB	95°C – 2 min 40 cycles at 95°C – 40 s 62°C – 30 s 72°C – 1 min 72°C – 7 min

Table 3.1 cont.

Primer and sequences (5'-3')	Target amplification	Thermocycling conditions
SdhC_F: ATGTTGGCACAGAAGCTCAC[6] SdhC_R: CTACAARAAMGCCAAMCCCAAC[6]	SdhC	95°C – 2 min 40 cycles at 95°C – 10 s 57°C – 20 s 72°C – 1 min 72°C – 4 min
SdhD_F: CGGGAATAACCAACCTCACT[3] SdhD_R: CCTCACTCCTCCAAACCGTA[3]	SdhD	95°C – 2 min 40 cycles at 95°C – 40 s 61°C – 30 s 72°C – 1 min 72°C – 7 min
Mg51P_F1: GTGGCGAGGGCTTGACTAC[4] Mg51P_R1: CGCGAGGACTTCCTGGA[4]	CYP51 promoter insert	94°C – 2 min 40 cycles at 94°C – 10 s 62°C – 30 s 72°C – 1.5 min 72°C – 8.5 min
MFS1_2F: GCAAGGATTCGGACTTGACG[5] MFS1_4R: CTGCCGGTATCGTCGATGAC[5]	*Mg*MFS1 promoter insert	95°C – 2 min 40 cycles at 95°C – 10 s 55°C – 20 s 72°C – 2 min 72°C – 8 min

[1]Primer used for sequencing only.
[2]Primer designed by Dubos *et al.* (2013).
[3]Primer designed by Dooley *et al.* (2016).
[4]Primer designed by Cools *et al.* (2012).
[5]Primer designed by Omrane *et al.* (2017).
[6]Primer designed by Fraaije, *B.* (unpublished).
[7]Primer designed during the current study.

3.2.4 Fungicide sensitivity test

Further sensitivity tests with additional specific compounds to those tested in Chapter 2 were carried out to characterise specific resistance mechanisms. Technical grade fungicides were purchased from Sigma-Aldrich and dissolved in dimethylsulphoxide (DMSO). Dilution series were made and 12 concentrations, including untreated, tested in Sabouraud liquid media (Sabouraud Dextrose Broth – Oxoid, Basingstoke, UK). The fungicides fentin chloride and isofetamid were diluted as follows, respectively: 10, 3.3, 1.1, 0.4, 1.2E-01, 4.0E-02, 1.4E-02, 4.6E-03, 1.5E-03, 5.0E-04 and 1.7E-04 µg/mL; 50, 20, 8.0, 3.2, 1.3, 5.1E-01, 2.0E-01, 8.2E-02, 3.3E-02, 1.3E-02 and 5.0E-03 µg/mL. All further methods to determine the sensitivity (EC_{50} in µg/mL) of each isolate were carried out as described in chapter 2.

3.2.5 Data analysis

The sequence reads for each isolate were assembled with the software Geneious v10.1.3 (Biomatters, Auckland, New Zealand) and aligned with the sequence data from the wild type strain IPO323 using the MAFFT plugin v7.308 (Katoh & Standley, 2013) in Geneious (Biomatters). The nucleotide sequences were translated to amino acid sequences for the study of CYP51, cytochrome b, β-tubulin, and SDH variants. The isolates and CYP51 variants were categorized according to their sensitivity to azole fungicides. The Resistance Factors (RFs) were calculated for each isolate by dividing their EC_{50} value by the average EC_{50} value of six azole sensitive reference strains. These references were the isolates IPO001, IPO323, IPO90012, A12/3B/8, MM20 and 4439 with known wild type CYP51 (Fraaije *et al.*, 2012). Isolates with Resistance Factor lower than 10 ($RF \leq 10$) were considered sensitive, whereas those with Resistance Factor higher than 100 ($RF \geq 100$) were insensitive, and isolates laying between these ($10 < RF < 100$) were categorized as moderately sensitive. The same system was used to categorize isolates accordingly to their sensitivity to SDHI fungicides, though the Resistance Factors were calculated by dividing the EC_{50} value of the respective strains by the average EC_{50} value of the five SDHI sensitive reference strains IPO001, IPO323, IPO90012, A12/3B/8 and 4439 (Fraaije *et al.*, 2012).

The synonymous substitutions, which do not affect the amino acid sequence of the protein, from the *CYP51* exon sequences, plus intron sequences and 89 bp upstream and 33 bp downstream of the gene, were considered to be the genetic background of the sequence, and therefore used for the study of genetic diversity and evolutionary history of the isolates. The software DnaSP v6.12.03 (Rozas *et al.*, 2017) was used to measure population differentiation with the G_{st} index (Nei, 1973) and genetic diversity analysis with the haplotype diversity, Hd (Nei, 1987), the nucleotide diversity, Pi (Nei, 1987) and the average number of nucleotide differences, K (Tajima, 1983).

The evolutionary analysis of the genetic background of the gene was studied with two different approaches. All 103 sequences (including the reference IPO323) were aligned using the MAFFT plugin in Geneious (Biomatters). Initially, the genealogy of haplotypes based on synonymous mutations was visualised in a haplotype network. This network was constructed with the statistical parsimony approach (TCS) (Clement et al., 2002) implemented in PopArt (Leigh & Bryant, 2015). An alternative approach was to reconstruct the phylogenetic relationship of haplotypes based on a likelihood analysis of *CYP51* genetic background. However, because recombination violates the assumption of one evolutionary history in the sequence dataset (Arenas & Posada, 2010), recombined haplotypes had to be identified and excluded from phylogenetic analysis.

Potential recombinants haplotypes were identified as described by Dooley (2015). At first, the whole sequences, including synonymous and non-synonymous mutations, were aligned using the MAFFT plugin in Geneious (Biomatters) and a TCS network was constructed in PopArt. A total of 41 haplotypes were generated. Reticulations in the network are evidence of homoplasy due to either independent mutation events or intragenic recombination (Brunner *et al.*, 2008). Furthermore, recombination breakpoints were identified using GARD (Kosakovsky Pond *et al.*, 2006), a Genetic Algorithm for Recombination Detection, implemented in the software found at datamonkey.org (Delport *et al.*, 2010). This analysis identified one recombination breakpoint (RBP) in the whole dataset at nucleotide

position 715. Phylogenetic reconstructions of sequence data from the left and right of the RBP were used to highlight potential recombinants that moved between clades.

After 16 potential recombinant haplotypes were removed the genetic backgrounds of the sequences were used for phylogenetic reconstruction. The TPM1uf+I nucleotide substitution model was selected by AIC in jModeltest v2.1.10 (Posada, 2008). The maximum likelihood (ML) tree was constructed using the PHYML plugin (Guindon *et al*., 2010) installed in Geneious (Biomatters), with the substitution model selected in jModeltest; starting tree with optimised topology, length and rate parameters; topology searching by the best of NNI and SPR, and 1000 bootstraps. The resulting maximum likelihood (ML) tree was condensed to branches with statistical support values over 70 using the software Mega X v.10.0.5 (Kumar *et al.*, 2018).

3.3 Results

3.3.1 Effects of CYP51 variants on azole sensitivity

The complete *Z. tritici CYP51* gene (*MgCYP51*) was amplified for a selection of field isolates for each of the 10 populations described in material and methods. A total of five primers were used to sequence one PCR amplicon, which encompassed the 1907 bp sequence containing three introns and four exons, alongside 89 bp upstream and 33 bp downstream of the gene (Figure 3.1).

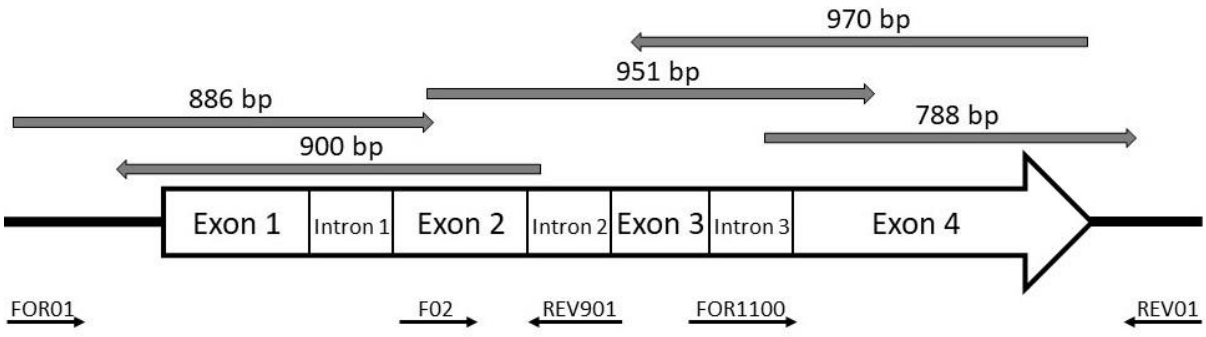

Figure 3.1 Schematic of strategy to amplify *Zymoseptoria tritici CYP51*.

Sequence comparisons between the translated CYP51 sequence from the reference isolate IPO323, with 544 predicted amino acid residues, and isolates of different azole sensitivities identified amino

acid substitutions and deletions correlated with azole resistance. A total of 21 amino acid alterations/deletions were found in 13 positions throughout the protein due to non-synonymous substitutions or deletions in the *CYP51* encoding gene. Altogether, 35 different CYP51 variants, including the wild type, were identified within 102 sequenced isolates. All variants were tested for azole sensitivity and then compared to six azole sensitive reference strains.

3.3.1.1 South American and reference populations

Two wild type isolates were found in South America, in Argentina and La Estanzuela (Table 3.2). All other isolates had alterations previously described elsewhere. The six-base pair (6 bp) deletion, leading to the loss of two amino acids (ΔY459/G460), was found in all populations sampled in South America. In addition, an insert of approximately 800 bp (~800 bp) in the promoter region of *CYP51* was found in a selection of isolates carrying one of two different CYP51 haplotypes.

In Chile, four different combinations of amino acid alterations were found across the spectrum of azole sensitivity. Epoxiconazole and tebuconazole sensitivity tended to decline with the increase of amino acid alterations in the CYP51 variants, but this was not observed with prochloraz (Figure 3.2). No CYP51 wild type was found in Chile, although the variant with alterations S188N & N513K showed the lowest EC_{50} values to epoxiconazole and tebuconazole of strains that were genotyped. The strain carrying this genotype presented Resistance Factors (RFs) ≤ 1, compared to the average EC_{50} value of six azole sensitive CYP51 wild type strains. The CYP51 variant V136A, S188N, Δ459 & Δ460, only found in Chile, had a moderate degree of sensitivity to epoxiconazole (RF = 23). This variant showed low sensitivity to prochloraz but high sensitivity to tebuconazole and strains with this genotype formed a cluster at the top left of Figure 3.2 B, not seen in any other population in South America. Another variant only found in Chile was L50S, S188N, Δ459, Δ460 & N513K, that was sensitive to epoxiconazole but only moderately sensitive to tebuconazole and prochloraz.

The strains with the highest EC_{50} values epoxiconazole in Chile had the L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K alterations in CYP51. Moreover, they were insensitive to tebuconazole but

sensitive to prochloraz (Figure 3.2). Three strains with this variant from Chile had an insert of approximately 800 bp in the CYP51 promoter region, whilst the same variant, when found in the other populations from South America, carried no insert. A noteworthy reduction in sensitivity to the three azole fungicides was observed in those strains from Chile with the insert (~800 bp) compared to the strain without an insert from this same population or the other three South American populations (Table 3.2). Interestingly, an overall negative correlation between tebuconazole and prochloraz sensitivity could be seen between the four groups of CYP51 variants, but a good positive correlation was observed within haplotypes (Figure 3.2 B).

In Argentina, Ruta 50 and La Estanzuela five variants were found with only one amino acid substitution (Figure 3.3, Figure 3.4 and Figure 3.5). These variants, Y459H, G460D, Y461S, Y461H, and Y461N, showed similar epoxiconazole sensitivity to the reference sensitive CYP51 wild type strains. Apart from Y459H, which was sensitive to all azoles, they had RF > 15 and RF > 6 to tebuconazole and prochloraz, respectively (Table 3.2). The two Uruguayan populations had strains with the variant L50S & Y461S sensitive to epoxiconazole but moderately sensitive to tebuconazole and prochloraz, similar to the single amino acid alteration Y461S.

Apart from Chile, in all South American populations, the CYP51 variant with alterations L50S, I381V & Y461H was the least sensitive to epoxiconazole. It was also insensitive to tebuconazole (RF = 165) and moderately sensitive to prochloraz (RF = 15.4) (Table 3.2). In general, this variant was the only one found in South America with a shift in sensitivity to all three azoles, forming a cluster of isolates with a phenotype of cross-resistance between prochloraz and tebuconazole in those three populations (Figure 3.3, Figure 3.4 and Figure 3.5). This CYP51 variant was only found associated with the ~800 bp insert in the promoter region, therefore it was not possible to assess whether the insert also had a role in reducing azole sensitivity. Ruta 50 presented a similar variant, L50S, I381V & Y459D, without the promoter insert but still with resembling RFs to L50S, I381V & Y461H in all azoles tested (Table 3.2).

The CYP51 variant L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K present in Argentina, Ruta 50 and La Estanzuela did not carry the ~800 bp insert and unlike in Chile this genotype was not the least sensitive to epoxiconazole, nor was it lower in sensitivity to tebuconazole when compared to L50S, I381V & Y461H (Figure 3.3, Figure 3.4 and Figure 3.5).

The isolates sampled in Rothamsted are a good representative of a European *Z. tritici* population carrying complex CYP51 variants. The nine different variants found within the 12 isolates sequenced from this population had higher RFs to epoxiconazole and prochloraz than those seen in South America, but the range of EC_{50} values overlapped for tebuconazole (Table 3.2). They all had four or more amino acid alterations and most of them carried the combination V136A-C/I381V/S524T alteration. Two different size inserts were found in the CYP51 promoter region, the 120 bp insert identified by Cools *et al.* (2012) was present in two strains with the L50S, S188N, I381V, Δ459, Δ460 & N513K variant and the ~800 bp previously described was present in all other sequenced strains, but one that had no PCR product.

The variant L50S, D134G, V136A, I381V & Y461H, existing in three isolates from Rothamsted had the lowest log_{10} EC_{50} values to the three azole fungicides, nonetheless, it was moderately sensitive and more complex compared to most South American genotypes since it carried together alterations V136A and I381V (Table 3.2).

Three other CYP51 variants (L50S, V136C, S188N, I381V, Y461H & S524T; L50S, V136A, I381V, Y461H & S524T, and V136C, I381V, Y461H & S524T) did not have the double deletion (ΔY459/G460), nevertheless, they carried the alterations V136A-C/I381V/S524T and presented a phenotype either insensitive or moderately sensitive across the three azole fungicides (Table 3.2).

The isolate sampled at Rothamsted with the highest EC_{50} values to epoxiconazole and prochloraz had L50S, V136A, S188N, A379G, I381V, Δ459, Δ460 & S524T, and in addition, showed an MDR phenotype, resulting in sensitivity losses of up to seven-fold compared to the same variant without the MDR phenotype (Figure 3.6 and Table 3.2). The phenotype and insert which have been shown to be

associated with MFS1 efflux overexpression (Omrane *et al.*, 2017) was also detected in the genotype L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K + MFS1 with the ~800 bp CYP51 promoter insert, which showed an increase in RFs of approximately 3, 2 and 10 folds to epoxiconazole, tebuconazole, and prochloraz, respectively, compared to this variant without MFS1 overexpression found in Chile (Table 3.2).

Strains with the 120 bp insert in the promoter region of the CYP51 encoding gene were found with the L50S, S188N, I381V, Δ459, Δ460 & N513K alterations, with insensitive phenotypes to all three azoles (Figure 3.6). This CYP51 variant was not found without the promoter insert, thus it was not possible to estimate the relative effect on azole sensitivity of target site overexpression versus amino acid alterations.

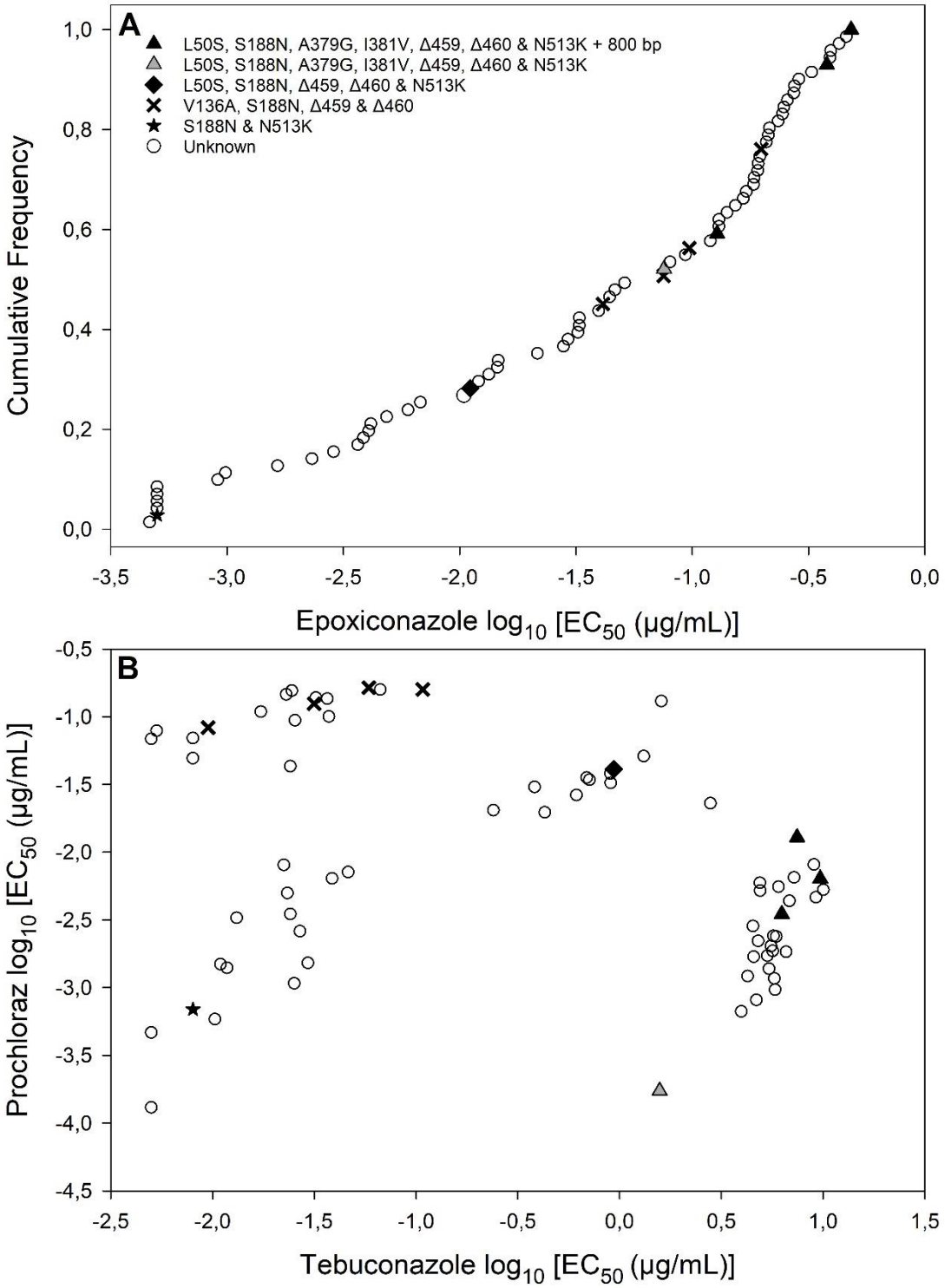

Figure 3.2 Sensitivity of CYP51 variants in *Zymoseptoria tritici* isolates from Chile to epoxiconazole (A), tebuconazole and prochloraz (B). S188N & N513K = Star; V136A, S188N, Δ459 & Δ460 = Cross; L50S, S188N, Δ459, Δ460 & N513K = Diamond; L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K = Gray Triangle Up; L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K + 800 bp = Black Triangle up.

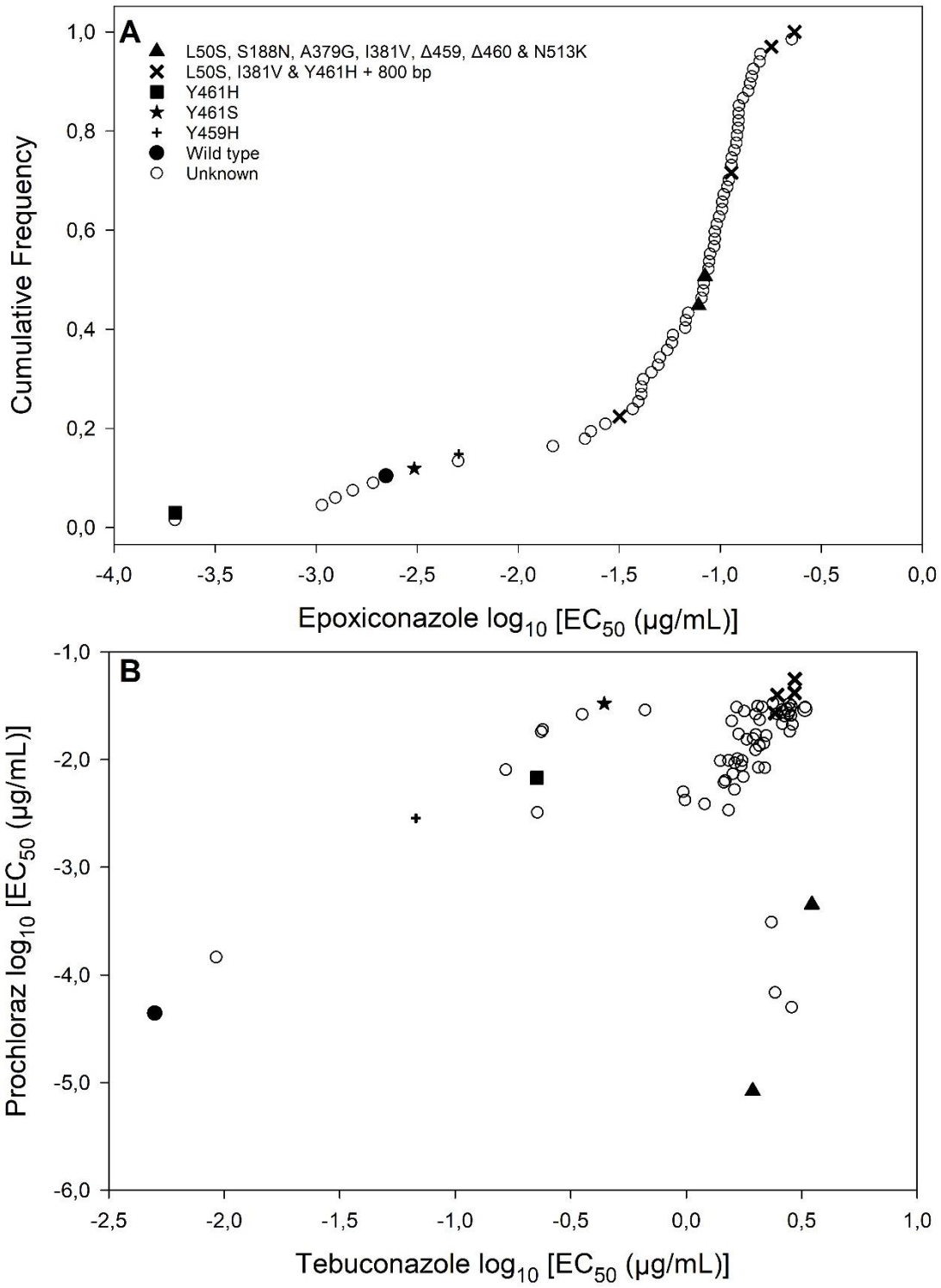

Figure 3.3 Sensitivity of CYP51 variants in *Zymoseptoria tritici* isolates from Argentina to epoxiconazole (A), tebuconazole and prochloraz (B). Wild type = Dark circle; Y459H = Plus; Y461S = Star; Y461H = Square; L50S, I381V & Y461H + 800 bp = Cross; L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K = Triangle up.

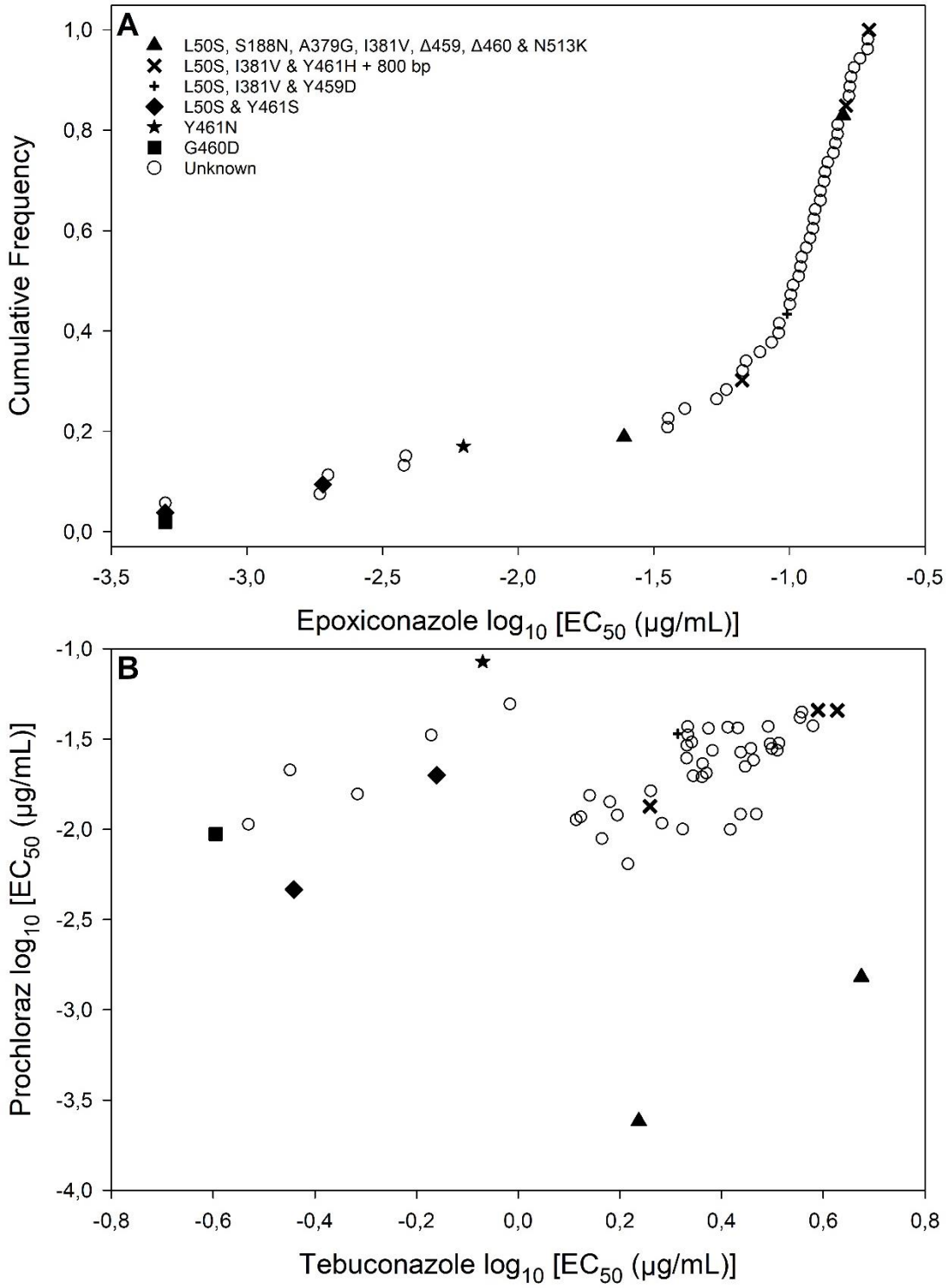

Figure 3.4 Sensitivity of CYP51 variants in *Zymoseptoria tritici* isolates from Ruta 50 - Uruguay to epoxiconazole (A), tebuconazole and prochloraz (B). G460D = Square; Y461N = Star; L50S & Y461S = Diamond; L50S, I381V & Y459D = Plus; L50S, I381V & Y461H + 800 bp = Cross; L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K = Triangle up.

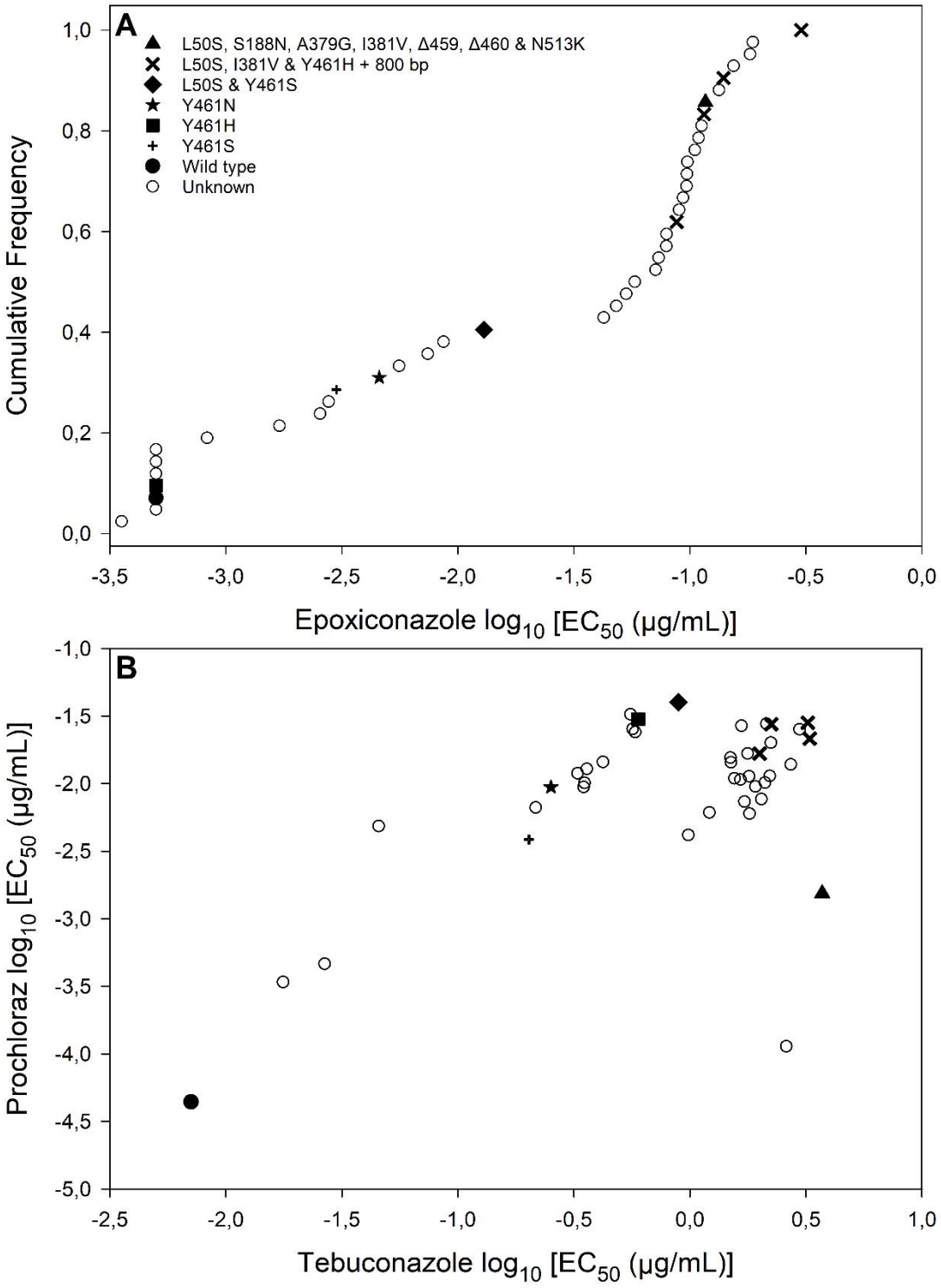

Figure 3.5 Sensitivity of CYP51 variants in *Zymoseptoria tritici* isolates from La Estanzuela - Uruguay to epoxiconazole (A), tebuconazole and prochloraz (B). Wild Type = Dark circle; Y461S = Plus; Y461H = Square; Y461N = Star; L50S & Y461S = Diamond; L50S, I381V & Y461H + 800 bp = Cross; L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K = Triangle up.

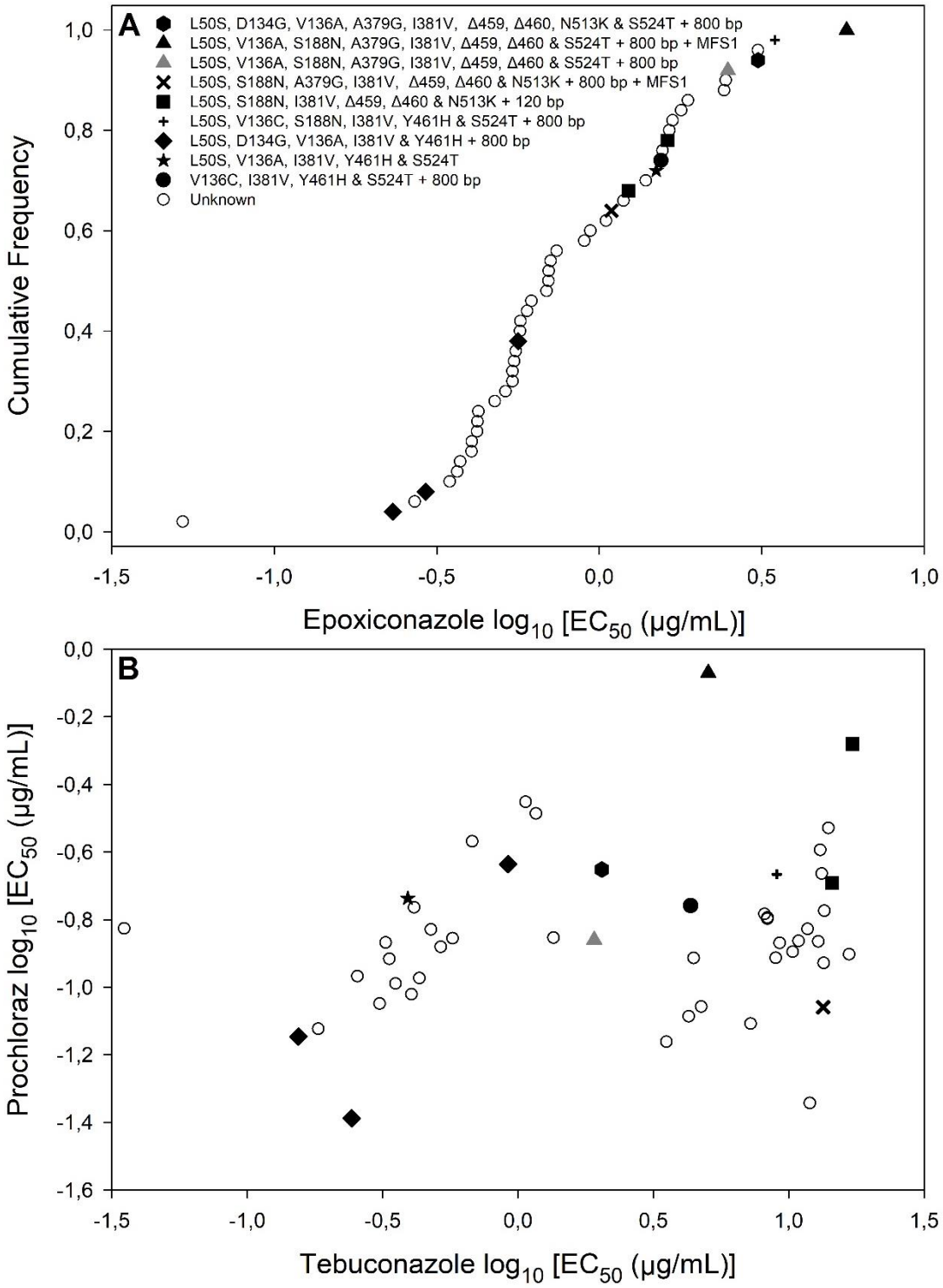

Figure 3.6 Sensitivity of CYP51 variants in *Zymoseptoria tritici* isolates from Rothamsted - UK to epoxiconazole (A), tebuconazole and prochloraz (B). V136C, I381V, Y461H & S524T + 800 bp = Dark circle; L50S, V136A, I381V, Y461H & S524T = Star; L50S, D134G, V136A, I381V & Y461H + 800 bp = Diamond; L50S, V136C, S188N, I381V, Y461H & S524T + 800 bp = Plus; L50S, S188N, I381V, Δ459, Δ460 & N513K + 120 bp = Square; L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K + 800 bp + MFS1 = Cross; L50S, V136A, S188N, A379G, I381V, Δ459, Δ460 & S524T + 800 bp = Gray Triangle up; L50S, V136A, S188N, A379G, I381V, Δ459, Δ460 & S524T + 800 bp + MFS1 = Black Triangle up; L50S, D134G, V136A, A379G, I381V, Δ459, Δ460, N513K & S524T + 800 bp = Hexagon.

3.3.1.2 Global populations

The North American populations, Michigan, and Oregon had primitive CYP51 variants compared to those from Rothamsted. No wild type sequences were found in North America, in contrast, the two amino acid deletions (ΔY459/G460) were not present in any strain sequenced. Although sampled in the same country, these populations did not share haplotypes. PCR of the CYP51 promoter region amplified the expected wild type product size in all isolates, therefore no inserts were present (Table 3.2).

One amino acid mutation from Michigan was novel, L144F, found in an isolate with a sensitive phenotype to all azoles. All haplotypes were sensitive to epoxiconazole and the less sensitive isolates showed positive cross-resistance to tebuconazole and prochloraz. Alterations V136A and I381V were not present in Michigan. Strains carrying amino acid alterations at positions Y459/Y461 had reduced sensitivity to tebuconazole and prochloraz (Figure 3.7).

Four different amino acid sequences were found in strains from Oregon. Isolates with alterations G460D and L50S & Y459D are sensitive to epoxiconazole and made part of a cluster of strains with positive cross-resistance between tebuconazole and prochloraz (Figure 3.8). Strains with L50S & Y459D are less sensitive to both fungicides and made the top of this cluster. Conversely, the haplotypes V136A & Y461S and I381V & Y461S were only moderately sensitive to epoxiconazole (Table 3.2) and showed negative cross-resistance between tebuconazole and prochloraz (Figure 3.8). Whilst V136A & Y461S was present in the strains least sensitive to prochloraz but most sensitive to tebuconazole, I381V & Y461S showed the opposite phenotype (Figure 3.8).

In Eastern European field populations of *Z. tritici* strains were found carrying modern CYP51 variants resembling those from Rothamsted-UK. There was neither wild type nor single amino acid alterations and most variants had the 6 bp deletion leading to the two amino acids loss (Δ459/G460) and in the case of a variant from Slovakia, a single amino acid deletion (ΔY459). Only two isolates from Slovakia had the ~800 bp insert in the promoter region of the CYP51 encoding gene, meanwhile, in Czech

Republic, two different size inserts were found in the CYP51 promoter region of different strains (Table 3.2).

The only isolate insensitive to epoxiconazole (RF ≥ 100) in Slovakia carried a modern CYP51 variant (L50S, V136A, S188N, A379G, I381V, Δ459, Δ460 & S524T) with the S524T amino acid alteration only seen in Rothamsted-UK and the ~800 bp CYP51 promoter insert (Figure 3.9 A). This same isolate was just moderately sensitive to tebuconazole and prochloraz (Table 3.2). The scatter plot between tebuconazole and prochloraz sensitivities grouped the strains in different clusters accordingly to CYP51 amino acid sequences. The haplotypes with L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K variant were insensitive to tebuconazole but sensitive to prochloraz, the opposite to a single strain with alterations D134G, V136A, S188N, Δ459 & Δ460. Interestingly both variants have similar epoxiconazole sensitivity. All other genotypes found in Slovakia formed a cluster with signs of a positive correlation between tebuconazole and prochloraz sensitivities (Figure 3.9).

The least sensitive variant from Czech Republic was L50S, S188N, I381V, Δ459, Δ460 & N513K. The strain carrying it also had the 120 bp insert in the promoter region of the CYP51 encoding gene and an insert in the *MFS1* promoter region. This isolate was resistant to the three azoles tested and showed an increase in RFs of 1.3, 1.4 and 2.5 folds to epoxiconazole, tebuconazole, and prochloraz, respectively, when compared to isolates of the same genotype from Rothamsted which did not overexpress MFS1 (Table 3.2 and Figure 3.10). The variant L50S, I381V & I461H was also present in the population from Czech Republic with and without the MDR phenotype. This variant is insensitive to tebuconazole, whereas it is moderately sensitive to epoxiconazole and prochloraz. The isolate with *MFS1* promoter insert showed RFs increased by 4.2, 3.2 and 5.6 fold to epoxiconazole, tebuconazole and prochloraz, accordingly, compared to isolates with the same CYP51 variant but without MFS1 overexpression (Table 3.2 and Figure 3.10).

As found in Chile, the CYP51 variant L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K was found in a strain from Czech Republic with the ~800 bp insert in the promoter region of the CYP51 encoding gene,

which was responsible for a significative reduction in azole sensitivity compared to another strain carrying this variant without a promoter insert (Figure 3.10). Interestingly, two strains carried both V136A and I381V together but without the two amino acids (ΔY459/G460) deletion. This variant (L50S, D134G, V136A, I381V & Y461H) was apparently widespread in the population from Rothamsted and those isolates showed a phenotype of moderate sensitivity to the three azoles. Even though L50S, D134G, V136A, I381V & Y461H was the most azole sensitive CYP51 variant in Rothamsted, in Czech Republic it was amongst the least sensitive to epoxiconazole and prochloraz (Table 3.2 and Figure 3.10).

A single CYP51 variant was found in New Zealand. This genotype had the amino acid alterations L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K, also seen in South America, Rothamsted-UK, and Eastern European populations. Seven out of 10 strains sequenced showed the presence of the ~800 bp insert in the *CYP51* promoter region. As in other populations, strains with the promoter insert showed a significant reduction in sensitivity to the three azoles, especially to prochloraz, compared to the three strains with wild type promoter sequence (Table 3.2 and Figure 3.11).

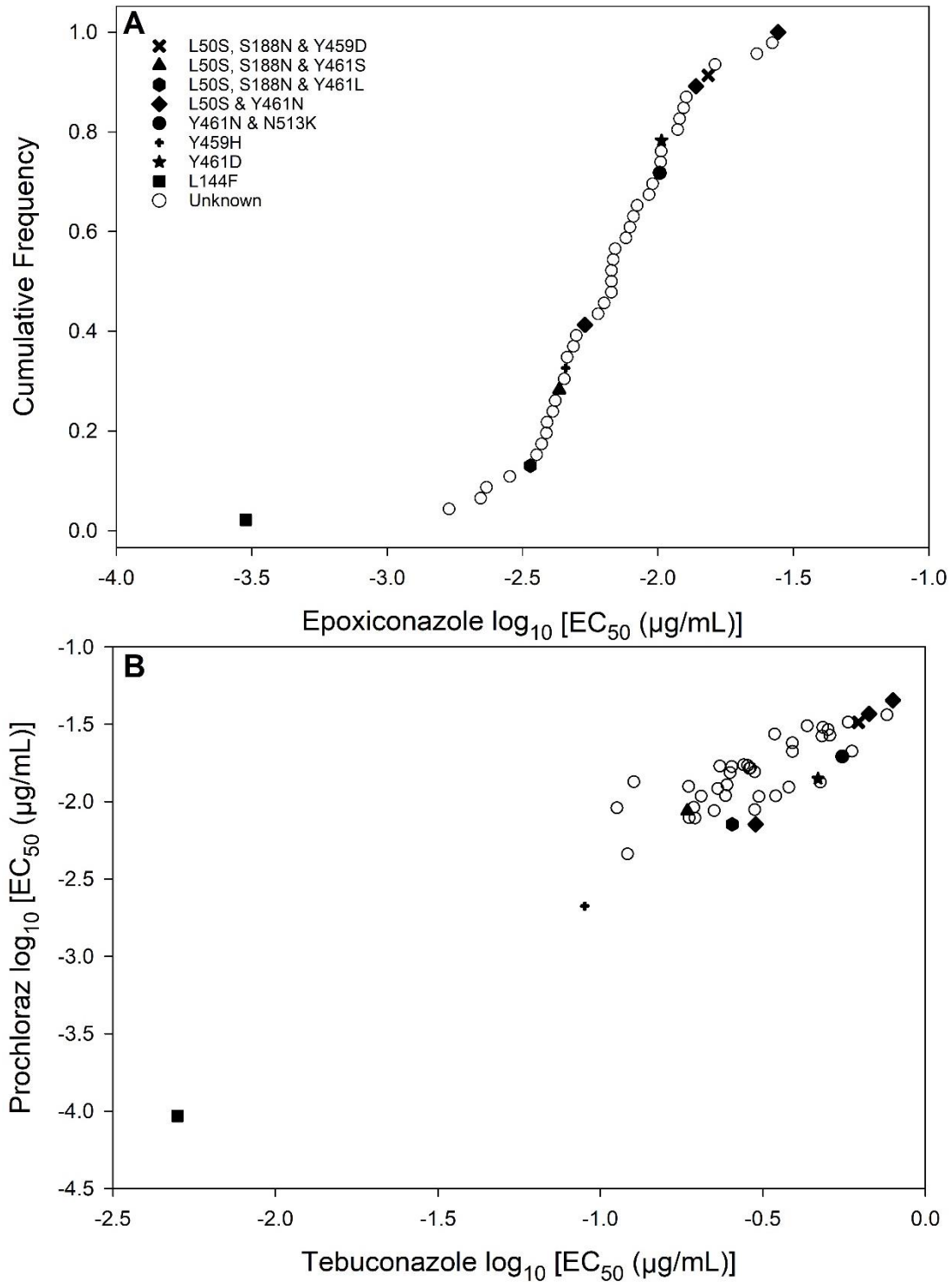

Figure 3.7 Sensitivity of CYP51 variants in *Zymoseptoria tritici* isolates from Michigan - USA to epoxiconazole (A), tebuconazole and prochloraz (B). L144F = Square; Y461D = Star; Y459H = Plus; Y461N & N513K = Dark circle; L50S & Y461N = Diamond; L50S, S188N & Y461L = Hexagon; L50S, S188N & Y461S = Triangle up; L50S, S188N & Y459D = Cross.

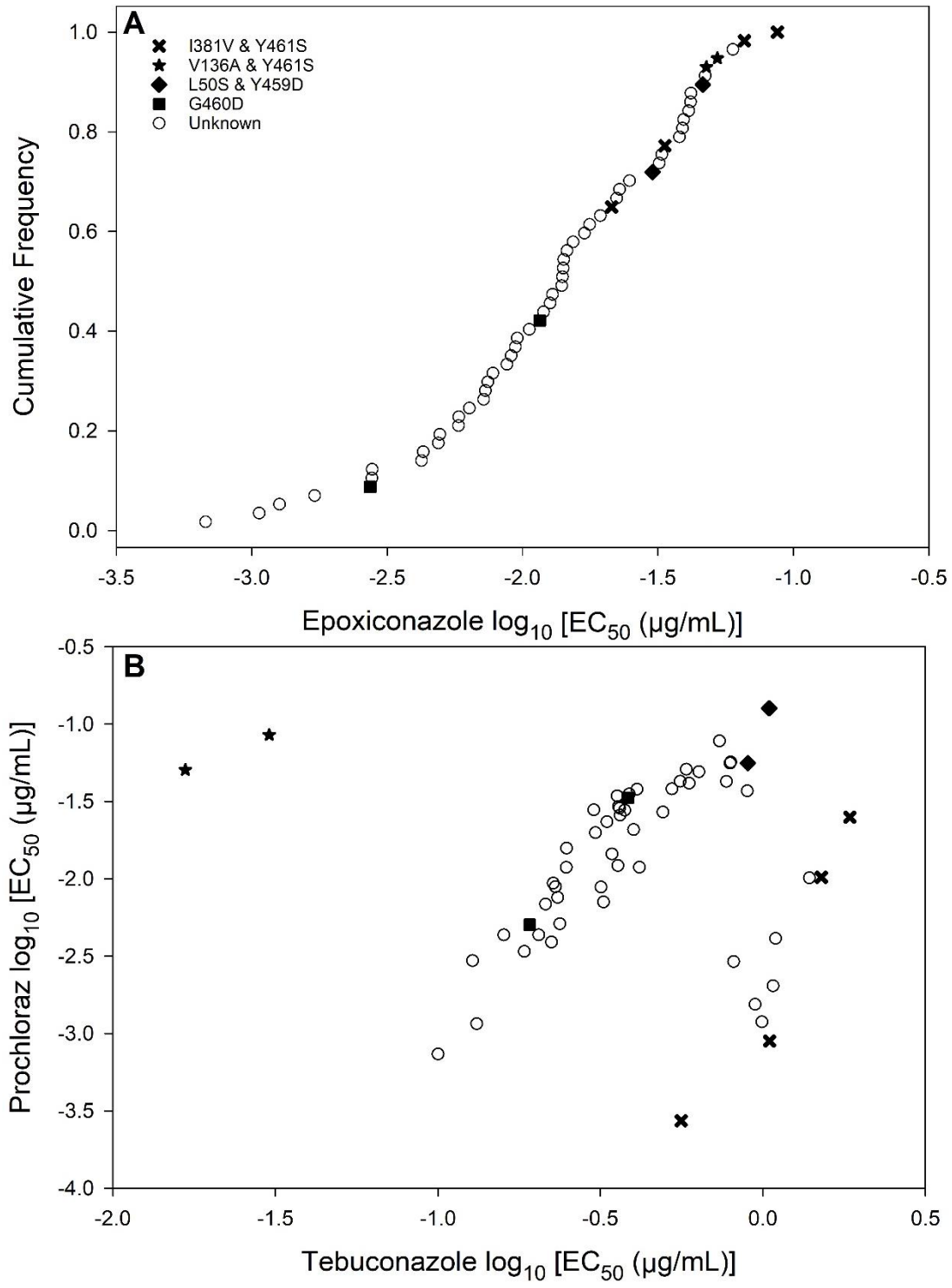

Figure 3.8 Sensitivity of CYP51 variants in *Zymoseptoria tritici* isolates from Oregon - USA to epoxiconazole (A), tebuconazole and prochloraz (B). G460D = Square; L50S & Y459D = Diamond; V136A & Y461S = Star; I381V & Y461S = Cross.

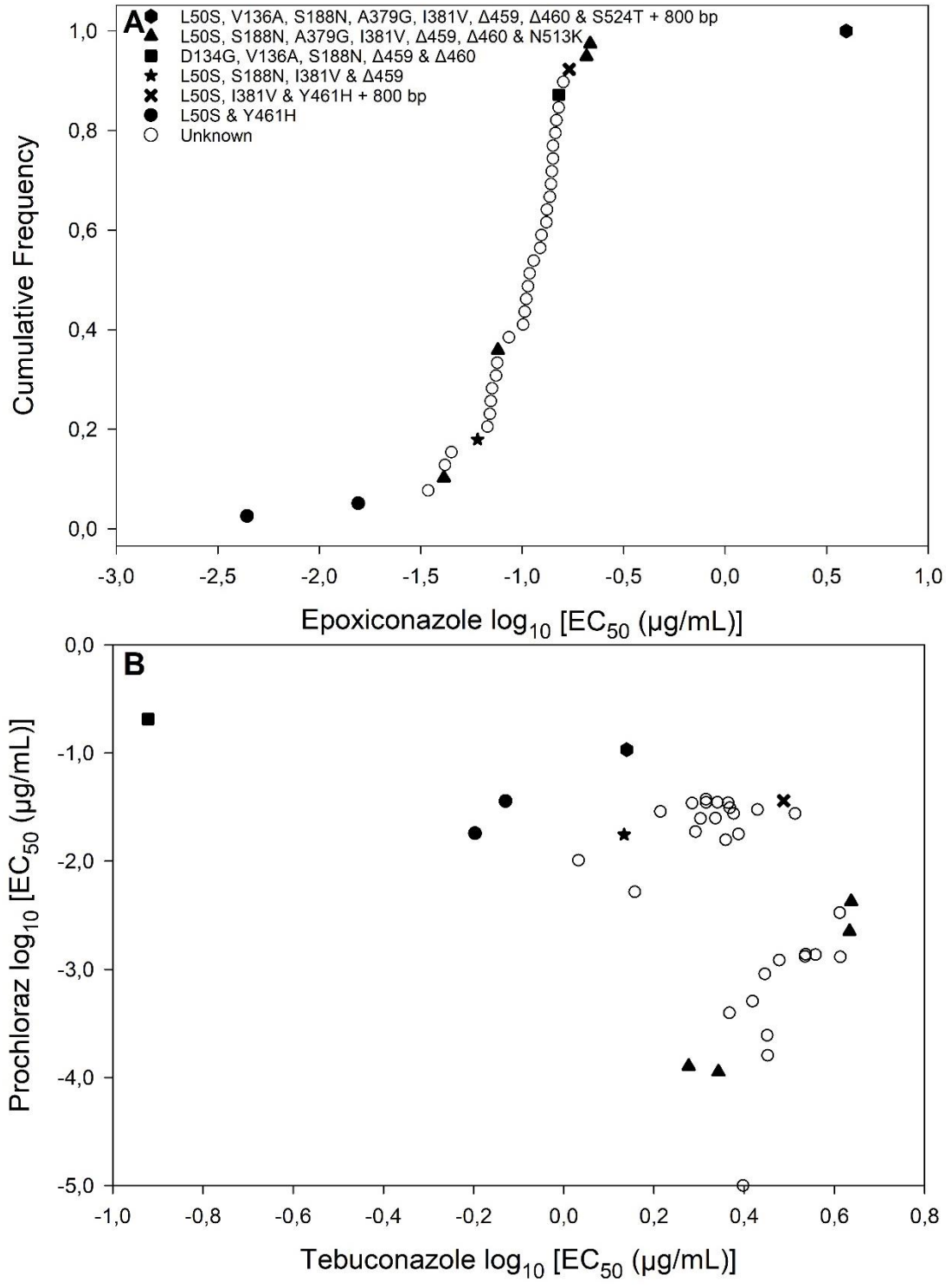

Figure 3.9 Sensitivity of CYP51 variants in *Zymoseptoria tritici* isolates from Slovakia to epoxiconazole (A), tebuconazole and prochloraz (B). L50S & Y461H = Dark circle; L50S, I381V & Y461H + 800 bp = Cross; L50S, S188N, I381V & Δ459 = Star; D134G, V136A, S188N, Δ459 & Δ460 = Square; L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K = Triangle up; L50S, V136A, S188N, A379G, I381V, Δ459, Δ460 & S524T + 800 bp = Hexagon.

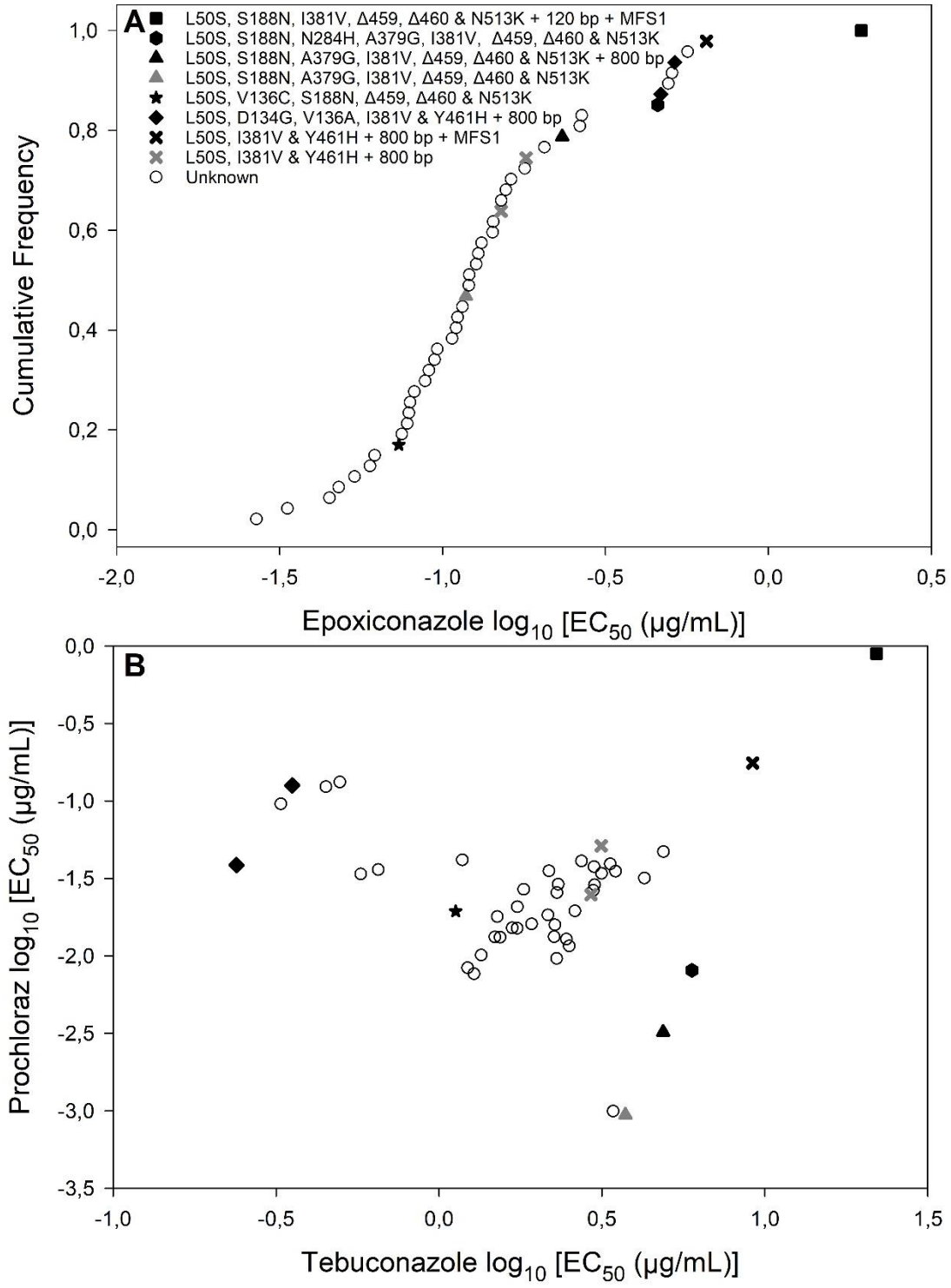

Figure 3.10 Sensitivity of CYP51 variants in *Zymoseptoria tritici* isolates from Czech Republic to epoxiconazole (A), tebuconazole and prochloraz (B). L50S, I381V & Y461H + 800 bp = Gray Cross; L50S, I381V & Y461H + 800 bp + MFS1 = Black Cross; L50S, D134G, V136A, I381V & Y461H + 800 bp = Diamond; L50S, V136C, S188N, Δ459, Δ460 & N513K = Star; L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K = Gray Triangle up; L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K + 800 bp = Black Triangle up; L50S, S188N, N284H, A379G, I381V, Δ459, Δ460 & N513K = Hexagon; L50S, S188N, I381V, Δ459, Δ460 & N513K + 120 bp + MFS1 = Square.

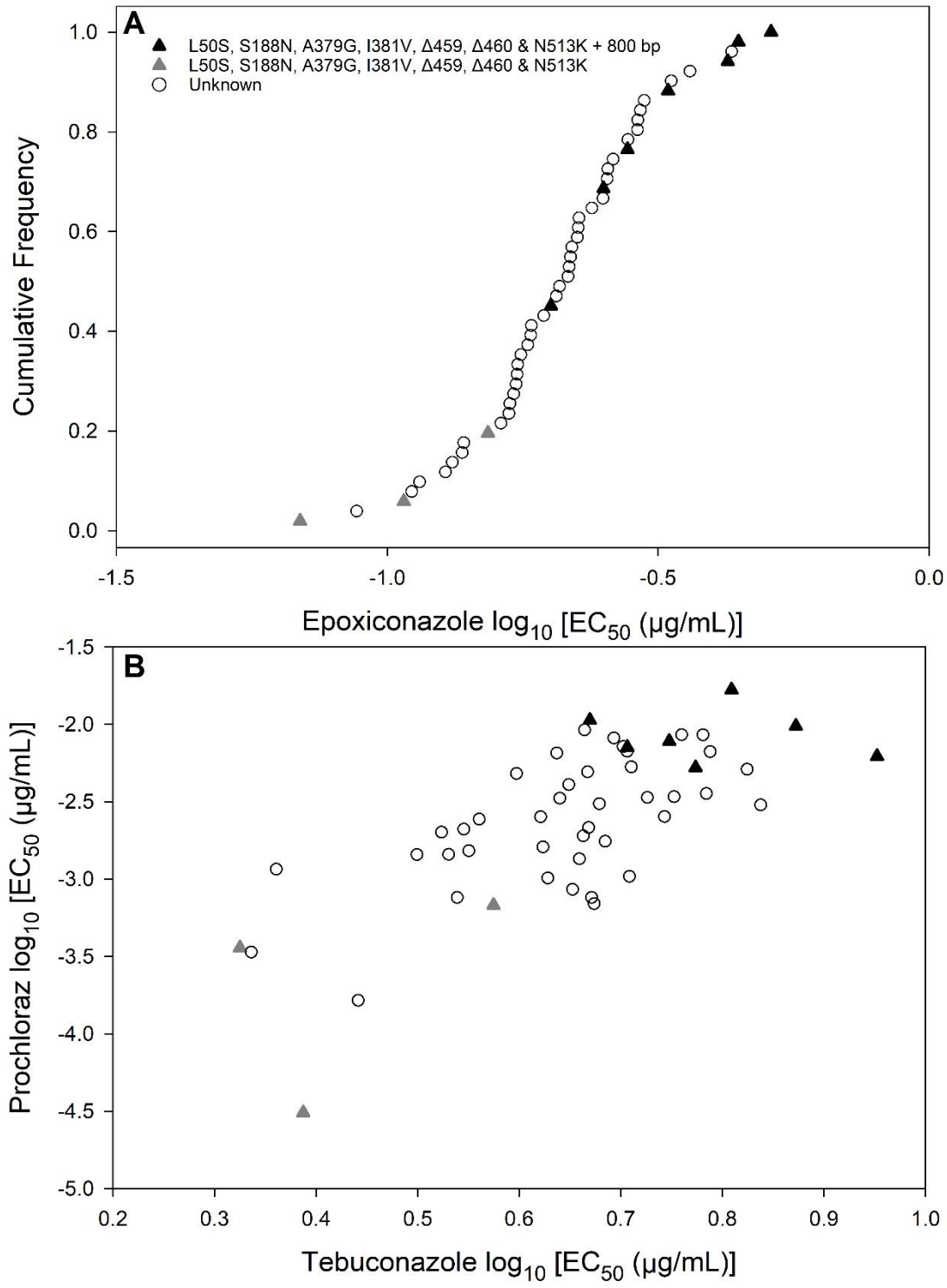

Figure 3.11 Sensitivity of CYP51 variants in *Zymoseptoria tritici* isolates from New Zealand to epoxiconazole (A), tebuconazole and prochloraz (B). L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K = Gray Triangle up; L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K + 800 bp= Black Triangle up.

3.3.2 Analysis of *CYP51* promoter inserts

Amplification of the region upstream of the CYP51 encoding gene generated three different amplicons. The wild type reference strain IPO323 yielded an amplicon of approximately 350 bp, which was presumably the 334 bp size fragment described by Cools *et al.* (2012). A larger fragment of approximately 450 bp was amplified in two isolates from Rothamsted and one isolate from the Czech Republic. This amplicon is consistent with a 120 bp insert linked to CYP51 overexpression (Cools *et al.*, 2012), only found in the same CYP51 haplotype [L50S, S188N, I381V, Δ459, Δ460 & N513K]. As presented above, a larger amplicon of approximately 1200 bp was found in different CYP51 backgrounds and indicates an insert of approximately 800 bp (~800 bp) (Table 3.2).

The CYP51 variant L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K was observed in Chile, Czech Republic, and New Zealand in strains either with the wild type amplicon length, or carrying the ~800 bp insert, in the *CYP51* promoter region. As mentioned above, isolates with the ~800 bp insert were on average 3-, 2- and 9-fold less sensitive to epoxiconazole, tebuconazole, and prochloraz, respectively (Table 3.2).

Sequencing of the larger amplicons in two strains from Chile and Rothamsted with CYP51 variant L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K identified an 862 bp insert located 205 bp upstream of the CYP51 encoding gene. Although located on the same site, the promoter insert sequenced in a New Zealand strain was in fact 787 bp long (Figure 3.12). The ~350 bp amplicon in the promoter of the strain from Chile with CYP51 variant L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K had 12 single-nucleotide mutations and a 7 bp insert compared to the predicted IPO323 promoter.

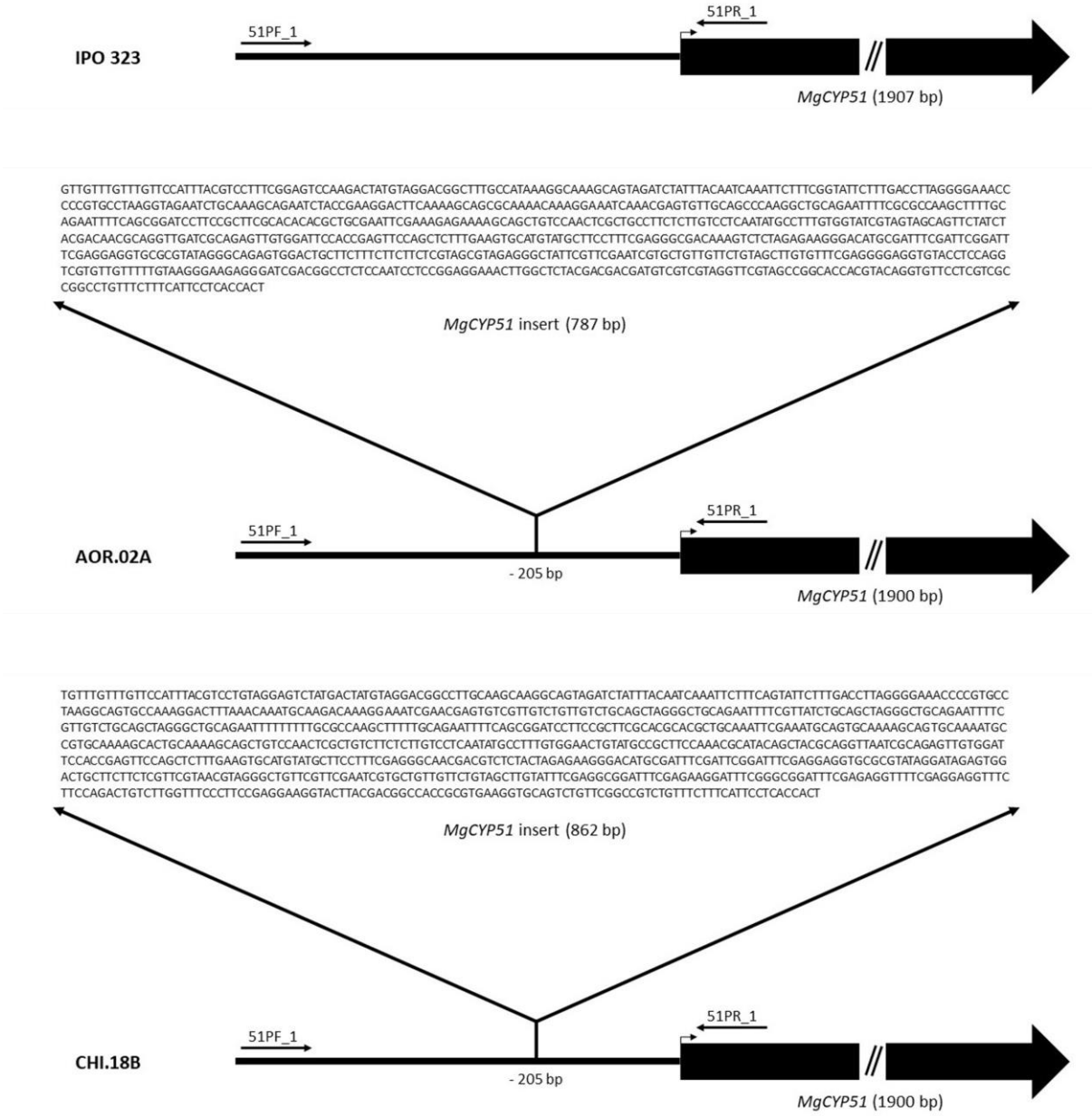

Figure 3.12 Schematic of strategy to amplify the region upstream of *MgCYP51* according to Cools *et al.* (2012) and the site and sequence of the inserts identified in isolates AOR.02A (New Zealand) and CHI.18B (Chile).

3.3.3 Analysis of *MFS1* promoter inserts

The discriminatory dose of 10 µg/mL of tolnaftate detected the Multiple-Drug-Resistance (MDR) phenotype in some *Z. tritici* field isolates from Oregon, Czech Republic, and Rothamsted and in the reference strain NT.321.17 (Table 3.3). These strains were tested with a PCR followed by sequencing with primers MFS1_2F and MFS1_4R (Table 3.1) to check if an *MFS1* promoter insert was linked to the MDR phenotype as described by Omrane *et al.* (2015).

Amplification of the 500 bp region upstream of the predicted translation starting codon of the MFS1 encoding gene yielded a fragment of approximately 450 bp in the wild type IPO323 and approximately 1000 bp in resistant reference isolate NT321.17, which when sequenced contained the expected 519 bp (type I) insert reported by Omrane *et al.* (2015). Within field isolates with the MDR phenotype, only the isolate RR.37 from Rothamsted-UK had one of the three inserts already described, the 339 bp insert (type II) identified in Omrane *et al.* (2017). Strains RR.19, from Rothamsted-UK, and CR.25, from Czech Republic, generated a 750 bp amplicon and sequencing showed a 267 bp insert, located in the same site as the type II insert (Figure 3.13). However, isolates with MDR phenotype from Oregon, ORE.47 and ORE.52, yielded amplicon fragments of similar size to IPO323 and further sequencing analysis showed no larger inserts in the *MFS1* promoter region. The *MFS1* promoter region in isolate CR.32 could not be amplified, probably due to sequence polymorphism.

Isolates with MDR phenotype were previously reported to have increased RFs of azole fungicides (Table 3.2). The 50% effective concentration (EC_{50} value) of fentin chloride and bixafen was determined for these field isolates and resistance factors were calculated relative to the IPO323 strain. Resistance factors of these fungicides are not correlated with *CYP51* alleles and are therefore a good indicator of efflux pump overexpression. The isolate NT321.17, with the 519 bp insert, had higher RFs for both fungicides, as did RR.37 with the 339 bp type II insert. Strains CR.25 and RR.19 carrying the 267 bp insert showed RFs of 8.57 and 7.43 to bixafen and 4.84 and 4.75 to fentin chloride, respectively.

Nevertheless, these RF values linked to the 267 bp insert were only slightly higher than those seen in strains from Oregon carrying no inserts (Table 3.3).

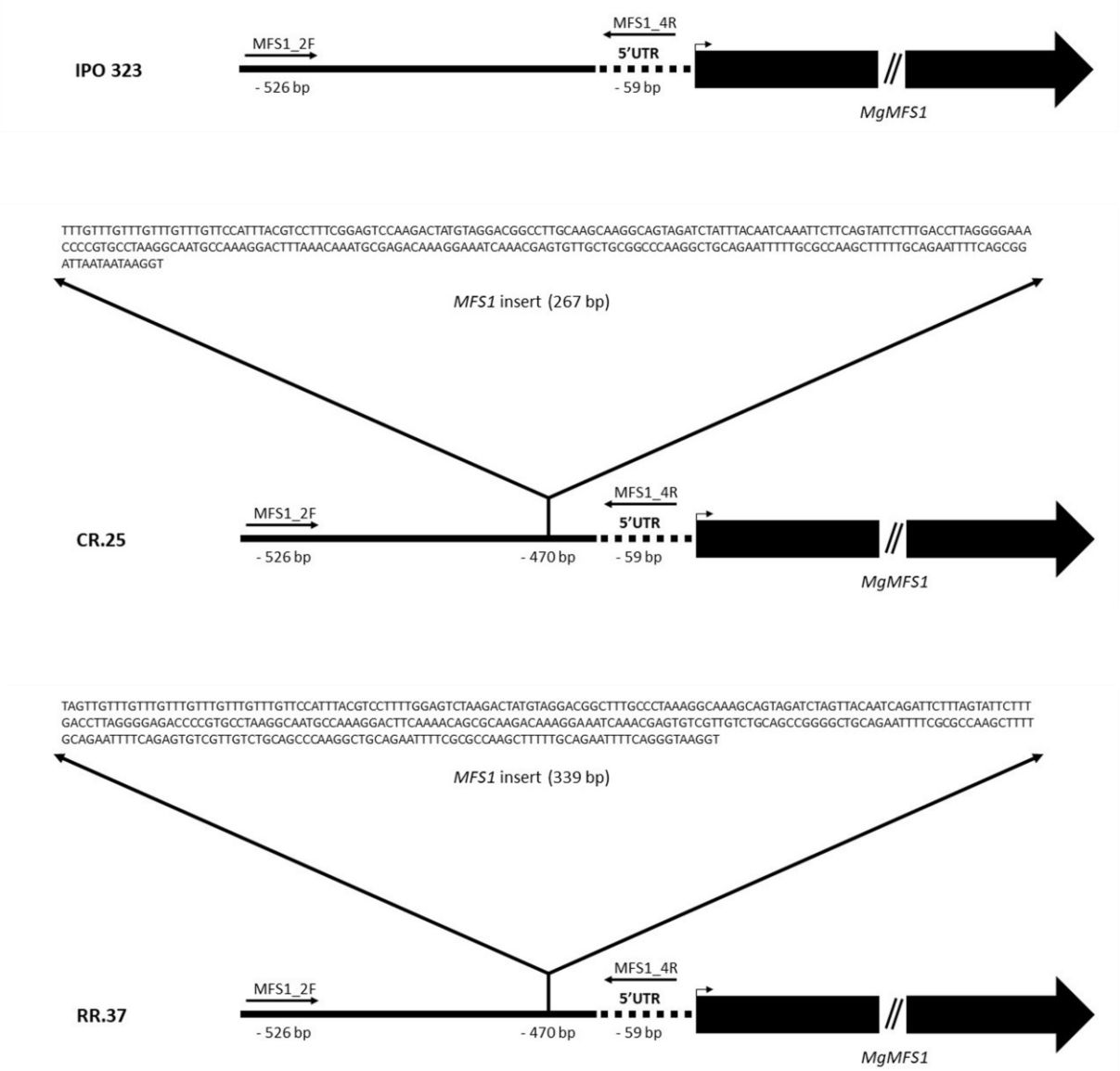

Figure 3.13 Schematic of strategy to amplify the region upstream of *MgMFS1* according to Omrane *et al*. (2017) and the site and sequence of the inserts identified in isolates CR.25 (Czech Republic) and RR.37 (Rothamsted-UK).

Table 3.2 Sensitivity of CYP51 variants to azole fungicides epoxiconazole, tebuconazole, and prochloraz. CYP51 variants were classified as sensitive = Green, moderately sensitive = Yellow and insensitive = Red.

CYP51 Variant[1]	n	Population[2]	Promoter Insert[3] (bp)	Epoxiconazole		Tebuconazole		Prochloraz	
				Log_{10} EC_{50} ± SE	RF[4]	Log_{10} EC_{50} ± SE	RF[4]	Log_{10} EC_{50} ± SE	RF[4]
Wild Type	6	References	Wild Type	-2.349 ± 0.001	-	-1.755 ± 0.006	-	-2.690 ± 0.001	-
Wild Type	2	ER, LE	Wild Type	-2.867 ± 8.6E-4	**0.30**	-2.219 ± 0.001	**0.34**	-4.356 ± 2.1E-8	**0.02**
L144F	1	MI	Wild Type	-3.523	**0.07**	-2.301	**0.28**	-4.031	**0.05**
Y459H	2	ER, MI	Wild Type	-2.317 ± 2.5E-4	**1.08**	-1.105 ± 0.011	**4.47**	-2.605 ± 3.7E-4	**1.22**
G460D	3	R50, OR	Wild Type	-2.307 ± 0.003	**1.10**	-0.558 ± 0.057	**15.8**	-1.797 ± 0.009	**7.81**
Y461S	2	ER, LE	Wild Type	-2.520 ± 2.9E-5	**0.68**	-0.492 ± 0.120	**18.4**	-1.733 ± 0.015	**9.06**
Y461H	2	ER, LE	Wild Type	-2.867 ± 8.6E-4	**0.30**	-0.385 ± 0.187	**23.5**	-1.736 ± 0.012	**8.98**
Y461D	1	MI	Wild Type	-1.987	**2.30**	-0.330	**26.6**	-1.852	**6.88**
Y461N	2	R50, LE	Wild Type	-2.265 ± 8.5E-4	**1.21**	-0.258 ± 0.300	**31.4**	-1.327 ± 0.038	**23.1**
S188N & N513K	1	CH	Wild Type	-3.301	**0.11**	-2.097	**0.46**	-3.161	**0.34**
Y461N & N513K	1	MI	Wild Type	-1.994	**2.27**	-0.255	**31.6**	-1.709	**9.58**

Table 3.2 cont.

CYP51 Variant	n	Population	Promoter Insert (bp)	Epoxiconazole		Tebuconazole		Prochloraz	
				Log_{10} EC_{50} ± SE	RF	Log_{10} EC_{50} ± SE	RF	Log_{10} EC_{50} ± SE	RF
L50S & Y459D	2	OR	Wild Type	-1.417 ± 0.008	**8.55**	-0.012 ± 0.073	**55.4**	-1.040 ± 0.035	**44.7**
L50S & Y461S	3	R50, LE	Wild Type	-2.290 ± 0.004	**1.15**	-0.188 ± 0.155	**36.9**	-1.667 ± 0.010	**10.5**
L50S & Y461H	2	SV	Wild Type	-2.000 ± 0.006	**2.23**	-0.161 ± 0.054	**39.3**	-1.569 ± 0.009	**13.2**
L50S & Y461N	3	MI	Wild Type	-1.806 ± 0.007	**3.50**	-0.230 ± 0.149	**33.5**	-1.527 ± 0.012	**14.6**
V136A & Y461S	2	OR	Wild Type	-1.301 ± 0.002	**11.2**	-1.629 ± 0.007	**1.34**	-1.168 ± 0.017	**33.2**
I381V & Y461S	4	OR	Wild Type	-1.284 ± 0.015	**11.6**	0.095 ± 0.281	**70.8**	-2.041 ± 0.006	**4.46**
L50S, S188N & Y459D	1	MI	Wild Type	-1.815	**3.42**	-0.206	**35.4**	-1.487	**15.9**
L50S, S188N & Y461S	1	MI	Wild Type	-2.365	**0.96**	-0.732	**10.6**	-2.061	**4.26**
L50S, S188N & Y461L	1	MI	Wild Type	-2.471	**0.76**	-0.594	**14.5**	-2.147	**3.49**
L50S, I381V & Y459D	1	R50	Wild Type	-1.008	**22.0**	0.314	**117**	-1.471	**16.5**
L50S, I381V & Y461H	14	ER, R50, LE, CR, SV	800	-0.818 ± 0.018	**34.0**	0.462 ± 0.187	**165**	-1.504 ± 0.004	**15.4**

Table 3.2 cont.

CYP51 Variant	n	Population	Promoter Insert (bp)	Epoxiconazole		Tebuconazole		Prochloraz	
				Log_{10} EC_{50} ± SE	RF	Log_{10} EC_{50} ± SE	RF	Log_{10} EC_{50} ± SE[2]	RF
L50S, I381V & Y461H + MFS1[5]	1	CR	800	-0.190	**144**	0.963	**523**	-0.755	**86.1**
V136C, I381V, Y461H & S524T	1	RR	800	0.190	**346**	0.638	**247**	-0.759	**85.3**
L50S, S188N, I381V & Δ459	1	SV	Wild Type	-1.220	**13.5**	0.134	**77.5**	-1.755	**8.61**
V136A, S188N, Δ459 & Δ460	4	CH	Wild Type	-0.988 ± 0.034	**23.0**	-1.286 ± 0.021	**2.95**	-0.877 ± 0.019	**65.0**
L50S, D134G, V136A, I381V & Y461H	5	CR, RR	800	-0.383 ± 0.065	**92.6**	-0.418 ± 0.138	**21.8**	-0.993 ± 0.036	**49.8**
L50S, V136A, I381V, Y461H & S524T	1	RR	ND[6]	0.175	**335**	-0.407	**22.3**	-0.737	**89.8**
L50S, S188N, Δ459, Δ460 & N513K	1	CH	Wild Type	-1.955	**2.48**	-0.029	**53.3**	-1.387	**20.1**
D134G, V136A, S188N, Δ459 & Δ460	1	SV	Wild Type	-0.819	**33.9**	-0.922	**6.82**	-0.686	**101**
L50S, V136C, S188N, I381V, Y461H & S524T	1	RR	800	0.541	**777**	0.956	**514**	-0.666	**106**
L50S, V136C, S188N, Δ459, Δ460 & N513K	1	CR	Wild Type	-1.134	**16.4**	0.051	**64.1**	-1.712	**9.51**
L50S, S188N, I381V, Δ459, Δ460 & N513K	2	RR	120	0.155 ± 0.194	**319**	1.199 ± 1.375	**901**	-0.439 ± 0.161	**178**
L50S, S188N, I381V, Δ459, Δ460 & N513K + MFS1[5]	1	CR	120	0.285	**431**	1.343	**1254**	-0.047	**439**

Table 3.2 cont.

CYP51 Variant	n	Population	Promoter Insert (bp)	Epoxiconazole		Tebuconazole		Prochloraz	
				Log_{10} EC_{50} ± SE	RF	Log_{10} EC_{50} ± SE	RF	Log_{10} EC_{50} ± SE^2	RF
L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K	14	CHI, ER, R50, LE, CR, SV, NZ	Wild Type	-0.963 ± 0.015	**24.4**	0.477 ± 0.298	**171**	-3.044 ± 3.1E-4	**0.44**
L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K	11	CHI, CR, NZ	800	-0.478 ± 0.038	**74.4**	0.819 ± 0.496	**375**	-2.090 ± 0.001	**3.98**
L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K + MFS1[5]	1	RR	800	0.037	**244**	1.127	**763**	-1.059	**42.7**
L50S, S188N, N284H, A379G, I381V, Δ459, Δ460 & N513K	1	CR	Wild Type	-0.339	**102**	0.776	**340**	-2.093	**3.95**
L50S, V136A, S188N, A379G, I381V, Δ459, Δ460 & S524T	2	SV, RR	800	0.508 ± 0.731	**720**	0.217 ± 0.267	**93.7**	-0.911 ± 0.015	**60.1**
L50S, V136A, S188N, A379G, I381V, Δ459, Δ460 & S524T + MFS1[5]	1	RR	800	0.762	**1291**	0.703	**287**	-0.070	**416**
L50S, D134G, V136A, A379G, I381V, Δ459, Δ460, N513K & S524T	1	RR	800	0.489	**688**	0.310	**116**	-0.652	**109**

[1]CYP51 variants are a combination of different amino acid alterations.
[2]Populations are CH = Chile, AR = Argentina, R50 = Ruta 50-UY, LE = La Estanzuela-UY, OR = Oregon-USA, MI = Michigan-USA, CR = Czech Republic, SV = Slovakia, NZ = New Zealand and RR = Rothamsted-UK.
[3]Insert of different lengths in the CYP51 promoter region (Wild Type, 120 bp and ~800 bp).
[4] Resistance Factors were calculated by dividing the EC_{50} value of the CYP51 variant with the average EC_{50} value of six azole sensitive CYP51 wild type strains.
[5]Multiple Drug Resistance (MDR) phenotype and presence of insert in the promoter region of MFS1.
[6]Not determined.

Table 3.3 Fungicide sensitivity of *Zymoseptoria tritici* field isolates and reference strains with different MFS1 alleles.

Isolate[1]	Population	MFS1 promoter insert (bp)	Bixafen			Fentin Chloride		
			Log_{10} EC_{50}	EC_{50}[2] (µg/mL)	RF[3]	Log_{10} EC_{50}	EC_{50} (µg/mL)	RF
ORE.47	Oregon	0	-0.474	0.336	**7.30**	-0.796	0.160	**3.57**
ORE.52	Oregon	0	-0.607	0.247	**5.37**	-1.043	0.091	**2.03**
CR.25	Czech Republic	267	-0.405	0.394	**8.57**	-0.664	0.217	**4.84**
CR.32	Czech Republic	ND[4]	-0.217	0.607	**13.2**	-0.819	0.152	**3.39**
RR.19	Rothamsted	267	-0.466	0.342	**7.43**	-0.671	0.213	**4.75**
RR.37	Rothamsted	339	-0.342	0.455	**9.88**	-0.519	0.303	**6.75**
IPO323	Reference	Wild Type	-1.337	0.046	**-**	-1.348	0.045	-
NT321.17	Reference	519	0.099	1.255	**27.3**	0.082	1.208	**26.9**

[1]All isolates, except for IPO323, showed MDR phenotype.
[2]EC_{50} values are the mean of two replicates.
[3]Resistance Factors were calculated by dividing the EC_{50} value of the respective strains by the EC_{50} value of sensitive IPO323 reference strain.
[4]Not determined.

3.3.4 Molecular mechanisms correlating with reduced QoI and MBC sensitivity

The 1173 bp *cytochrome b* sequence was amplified from a selection of isolates with different QoI sensitivities. These sequences were aligned to the wild type *cytochrome b* sequence from IPO323. Two amino acid substitutions, found in different isolates, were correlated with shifts in azoxystrobin sensitivity compared to the mean EC_{50} of six QoI sensitive references: The substitution TTC to TTA led to amino acid alteration F129L while GGA to GCA resulted in amino acid substitution G143A, both previously reported in QoI resistant *Z. tritici* (Figure 3.14).

The amino acid substitution G143A was detected in strains with azoxystrobin log_{10} EC_{50} higher than zero (EC_{50} > 1 µg/mL), whereas F129L was only present in two New Zealand strains with log_{10} EC_{50} of -0.118 and -0.248 (EC_{50} of 0.762 µg/mL and 0.565 µg/mL, accordingly). All sequenced strains with

azoxystrobin log_{10} EC_{50} less than -0.45 had the wild type *cytochrome b* encoding gene (Table 3.4). The *cytochrome b* genotype of each strain was inferred as Wild Type, F129L or G143A according to the azoxystrobin EC_{50} value for the strain (Figure 3.15). While the F129L mutation was only detected in New Zealand, G143A was widespread in all populations. All strains from Rothamsted had G143A genotype, however, in Chile, the wild type *cytochrome b* was the most common (80%), in contrast to the other three populations sampled in South America. The populations within North America and Eastern Europe also had contrasting frequencies of alleles. Oregon and Czech Republic had more strains with the resistant allele, in contrast in Michigan and Slovakia the wild type was at higher frequency (Figure 3.15).

A *β-tubulin* sequence of approximately 600 bp was amplified, using a pair of primers annealing within exon 6 of the predicted β-tubulin encoding gene, from a selection of *Z. tritici* field isolates with known phenotypes for resistance or sensitivity to MBC fungicides (Figure 3.16). This gene portion contains residues 198, and therefore sequencing this region can detect the allele E198A associated with MBC resistance. The partial *β-tubulin* sequences identified at codon position 198 the non-synonymous mutation GCG encoding Alanine (A) in carbendazim resistant isolates and the wild type sequence GAG encoding Glutamic acid (E) in carbendazim sensitive phenotypes (Figure 3.17). Other than this polymorphism, five synonymous polymorphisms were identified in the partial sequence.

The β-tubulin alteration E198A was identified in all isolates with the MBC resistant phenotype, that is, those strains which showed yeast-like growth in the presence of 1.0 µg/mL of carbendazim, while wild type β-tubulin was found in all sequenced carbendazim-sensitive isolates (Table 3.4).

Although varying in frequency, carbendazim resistant allele E198A appeared to be widespread around the world, present in all populations but Michigan. In the reference population Rothamsted, it was present in 94% of the strains. In South America, it was in higher frequency in Chile and Ruta 50, with 75 and 77% of E198A genotypes, respectively. Populations from Oregon and New Zealand were most sensitive, showing only 23 and 8% of E198A mutated strains, respectively. In contrast, Eastern

European populations had a resistant allele frequency (Slovakia = 74%; Czech Republic = 100%) as high as the reference population (Figure 3.18).

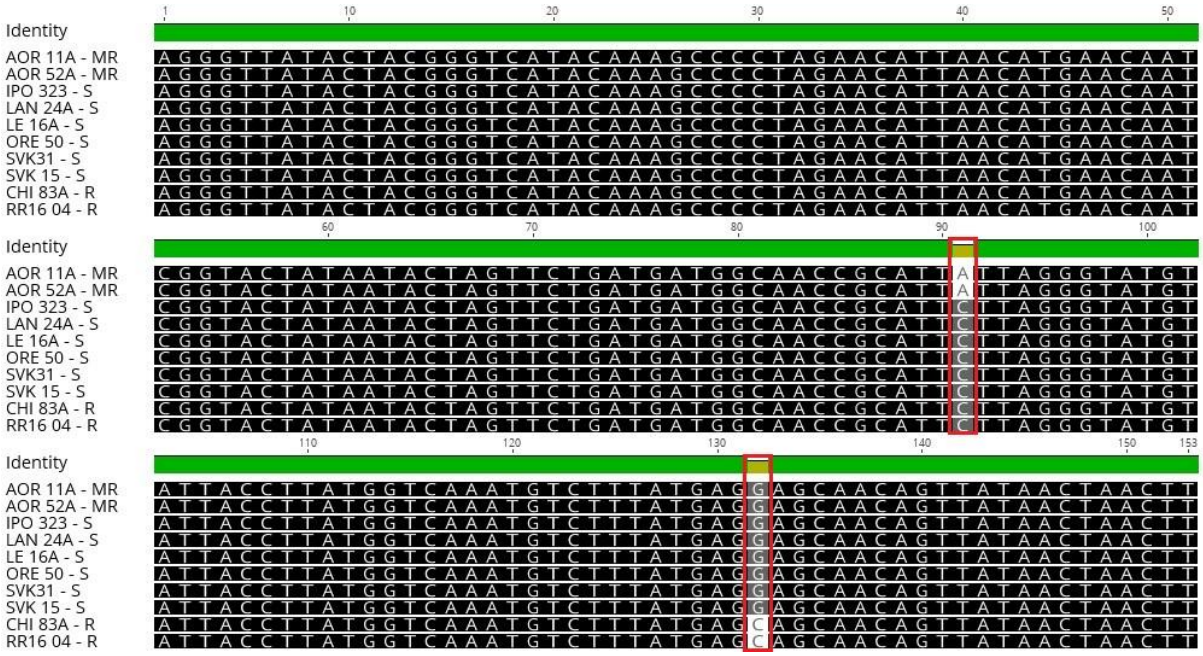

Figure 3.14 Alignment of partial cytochrome b sequences from 10 *Zymoseptoria tritici* field isolates. Amino acid substitutions correlated to QoI sensitivity are highlighted in red. Letters after isolate names designate azoxystrobin sensitivity: S = Sensitive (log_{10} EC_{50} < -0.45), MR = Moderately Resistant (-0.45 < log_{10} EC_{50} < 0) and R = Resistant (log_{10} EC_{50} > 0).

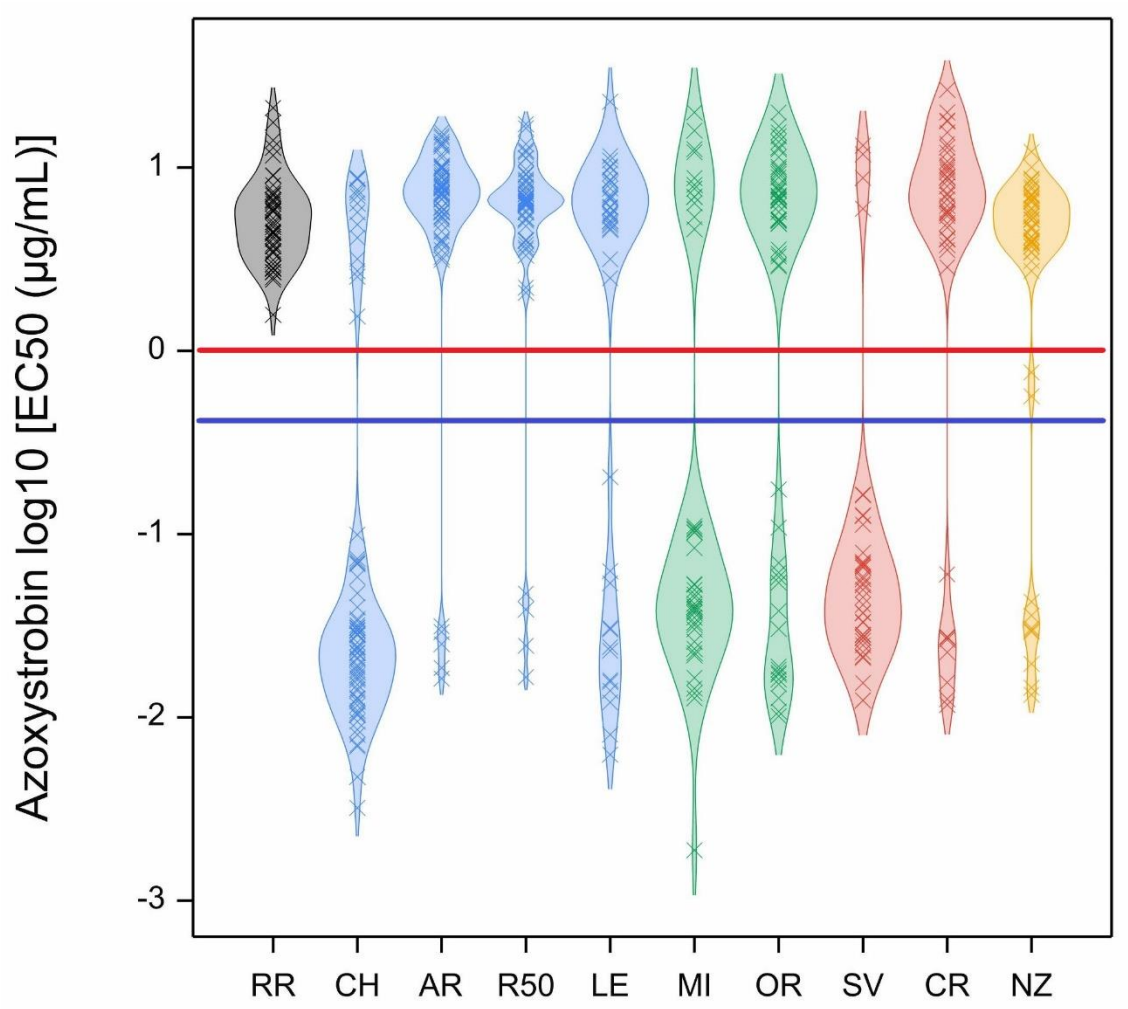

Figure 3.15 Frequency of cytochrome b genotypes in *Zymoseptoria tritici* field populations from Rothamsted-UK = Gray (RR), South America = Blue (Chile = CH, Argentina = AR, Ruta 50-UY = R50 and La Estanzuela-UY = LE), North America = Green (Michigan = MI and Oregon = OR), Eastern Europe = Red (Slovakia = SV and Czech Republic = CR) and New Zealand = Yellow (NZ). Wild type genotypes had log_{10} EC_{50} < -0.45 (under blue line), F129L alteration had -0.45 < log_{10} EC_{50} < 0 (between blue and red line) and G143A alteration had log_{10} EC_{50} > 0 (above red line).

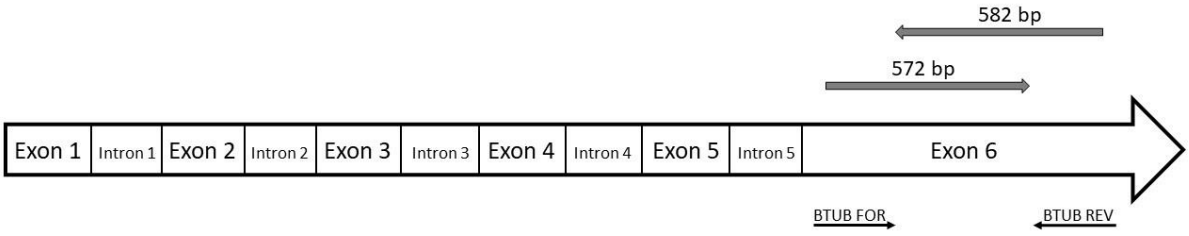

Figure 3.16 Schematic of strategy to amplify and sequence *Zymoseptoria tritici* partial *β tubulin*.

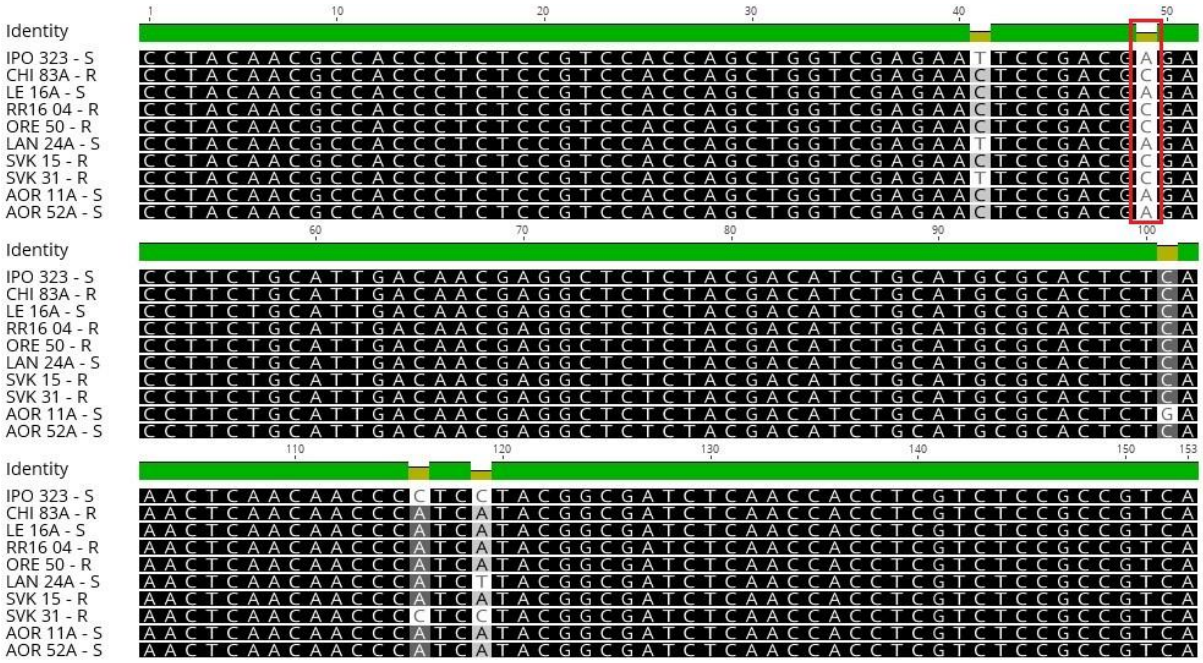

Figure 3.17 Alignment of partial *β-tubulin* sequences from 10 *Zymoseptoria tritici* field isolates. Amino acid substitutions correlated to MBC sensitivity are highlighted in red. Letters after isolate names designate carbendazim resistance measured by mycelial growth in the presence of 1.0 µg/mL of carbendazim: S = Sensitive (No growth) and R = Resistant (Normal Growth).

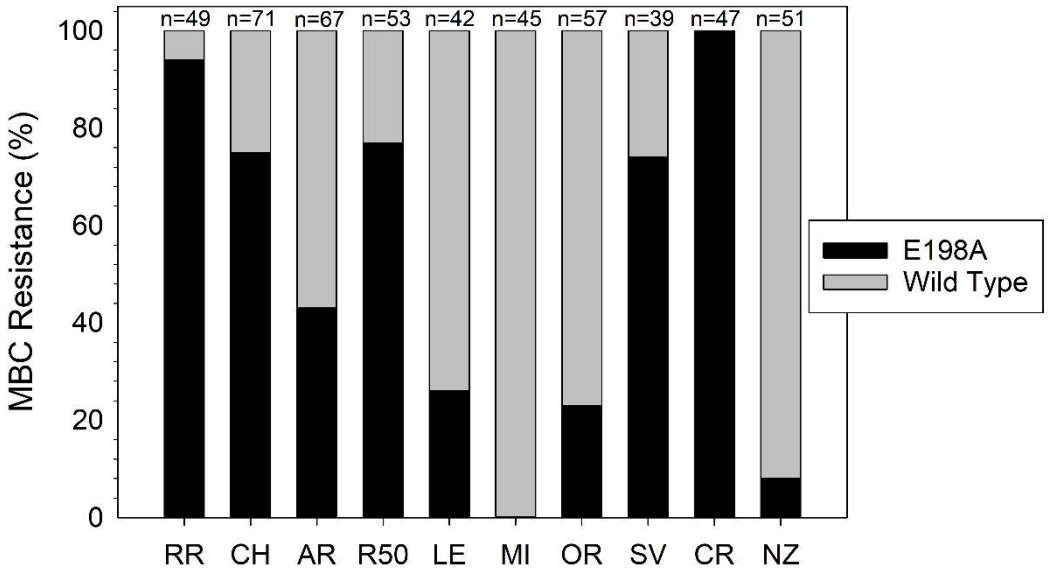

Figure 3.18 Frequency of carbendazim (MBC) resistant genotypes in *Zymoseptoria tritici* field populations from Rothamsted-UK (RR), Chile (CH), Argentina (AR), Ruta 50-UY (R50), La Estanzuela-UY (LE), Michigan-USA (MI), Oregon-USA (OR), Slovakia (SV), Czech Republic (CR) and New Zealand (NZ).

Table 3.4 Azoxystrobin and carbendazim sensitivity and associated genotype of selected *Zymoseptoria tritici* field isolates.

Isolate	Population	Azoxystrobin				Carbendazim	
		Cytochrome b genotype	Log_{10} EC_{50}	EC_{50}[1] (µg/mL)	RF[2]	β-tubulin genotype	Phenotype[3]
RR16.04	Rothamsted	G143A	0.197	1.572	**92.0**	E198A	**R**
CHI.02A	Chile	Wild Type	-2.325	0.005	**0.28**	Wild Type	**S**
CHI.38A	Chile	G143A	0.689	4.886	**286**	E198A	**R**
CHI.83A	Chile	G143A	0.188	1.542	**90.2**	E198A	**R**
ER.13A	Argentina	G143A	0.651	4.479	**262**	E198A	**R**
ER.58A	Argentina	Wild Type	-1.731	0.019	**1.09**	Wild Type	**S**
ER.61A	Argentina	G143A	1.054	11.33	**663**	Wild Type	**S**
R50.06	Ruta 50	G143A	0.317	2.075	**121**	E198A	**R**
R50.08C	Ruta 50	G143A	0.681	4.798	**281**	E198A	**R**
R50.47C	Ruta 50	G143A	0.758	5.724	**335**	Wild Type	**S**
LE.16A	La Estanzuela	Wild Type	-0.689	0.205	**12.0**	Wild Type	**S**
LE.21	La Estanzuela	G143A	0.750	5.627	**329**	Wild Type	**S**
LE.31A	La Estanzuela	Wild Type	-1.202	0.063	**3.67**	E198A	**R**
LAN.05A	Michigan	G143A	0.905	8.040	**470**	Wild Type	**S**
LAN.24A	Michigan	Wild Type	-0.740	0.182	**10.7**	Wild Type	**S**
ORE.01	Oregon	G143A	0.516	3.278	**192**	Wild Type	**S**
ORE.50	Oregon	Wild Type	-0.756	0.175	**10.3**	E198A	**R**
SVK.15	Slovakia	Wild Type	-0.783	0.165	**9.64**	E198A	**R**
SVK.31	Slovakia	Wild Type	-0.788	0.163	**9.54**	E198A	**R**
CR.25	Czech Republic	G143A	1.301	20.00	**1170**	E198A	**R**
AOR.11A	New Zealand	F129L	-0.118	0.762	**44.6**	Wild Type	**S**
AOR.51A	New Zealand	G143A	0.540	3.466	**203**	E198A	**R**
AOR.52A	New Zealand	F129L	-0.248	0.565	**33.0**	Wild Type	**S**

[1]EC_{50} values are the mean of two replicates.

[2]Resistance Factors were calculated by dividing the EC_{50} value of the respective strains by the average EC_{50} value of six QoI sensitive reference strains.

[3]Phenotypes were categorized accordingly to mycelial growth in the presence of 1.0 µg/mL of carbendazim as S = Sensitive (No growth) and R = Resistant (Normal growth).

3.3.5 Variation in *Sdh* genes and sensitivity to SDHI fungicides

Succinate dehydrogenase subunits B, C, and D encoding gene sequences were amplified in a selection of isolates less sensitive to bixafen and fluopyram from each of the populations. Sequences were aligned to the reference strain IPO323, then amino acid substitutions were determined and correlated to resistance factors (RFs) for each fungicide (Table 3.5).

None of the isolates selected had amino acid variants in subunit D. One isolate from Oregon carried a mutation causing the amino acid substitution I44V on Sdh-B, along with C-I29V. Resistance factors relative to reference strains in this isolate were 7.05 to bixafen and 81.9 to fluopyram. Although less sensitive to bixafen than the reference isolates, this bixafen EC_{50} was within the range of other strains with wild types Sdh-B and Sdh-C. However, the isolate was moderately insensitive to fluopyram and outside the range of the isolates with the reference sequence (Table 3.5).

From 29 isolates, a total of 22 showed at least one alteration in the amino acid sequence of Sdh-C. The most common haplotype was N33T & N34T, which appeared in 14 isolates across all populations but Czech Republic. In isolates with this genotype, RFs varied from 0.84 to 10.5 to bixafen and from 1.71 to 54.5 to fluopyram. One strain from Czech Republic had the single amino acid alteration N33T. The strain was sensitive to bixafen but moderately sensitive to fluopyram (Table 3.5).

Four isolates from North America had a single alteration C-I29V. They were sensitive to bixafen, with RFs ranging from 0.62 to 7.05. However, two strains from Oregon were insensitive to fluopyram, presumably due to changes elsewhere in the genome. This mutation was also found in combination with C-T79N, in Rothamsted, and C-F23S, in Slovakia. Both isolates were sensitive to both fungicides. Another isolate, from Czech Republic, carried C-Q42E, with a phenotype sensitive to bixafen but moderately sensitive to fluopyram (Table 3.5).

In this collection of isolates, bixafen sensitivity did not decrease more than 10.5-fold relative to the SDHI sensitive references, and the highest EC_{50} value was seen in NT321.17, which did not carry amino acid alterations in any of the Sdh subunits. Although some strains showed greater loss of sensitivity to

fluopyram, none of the target site mutations appeared to be correlated with shifts in sensitivity. Therefore, the isolates with the highest EC_{50} values to fluopyram in each population were tested with isofetamid, a SDHI fungicide known for positive cross-resistance to fluopyram but not bixafen, and fentin chloride, to test for Multiple Drug Resistance (MDR) phenotype (Table 3.6).

Most strains showed cross-resistance between fluopyram (RF > 10) and isofetamid (RF > 10). None of them showed signs of MDR since RFs for fentin chloride only stretched from 0.69 to 3.56, while NT321.17, known to overexpress the MFS1 efflux pump, had an RF of 26.9 (Table 3.6). This reference is also resistant to the SDHI fungicides, but at a lower level compared to the resistant strains mentioned above.

In general, none of the populations had bixafen highly resistant strains correlated to *Sdh* mutations. Some populations had a few strains slightly resistant to bixafen, with EC_{50} values only just above the cut-off value of 0.4 µg/mL (10 folds IPO323 EC_{50} value) (Table 3.7). In contrast, fluopyram slightly resistant isolates were widespread, while Rothamsted, Michigan and Czech Republic had approximately two per cent of strains with highly resistant phenotypes to this compound. Only Chile had no fluopyram resistant isolates. Interestingly, Oregon showed a much higher percentage of slightly and highly resistant phenotypes than other populations (Table 3.7).

Table 3.5 Bixafen and fluopyram sensitivity and associated genotype of selected *Zymoseptoria tritici* field isolates. Strains were classified as sensitive = Green, moderately sensitive = Yellow and insensitive = Red.

Isolate	Population	SdhB Genotype	SdhC Genotype	SdhD Genotype	Bixafen			Fluopyram		
					Log_{10} EC_{50}[1]	EC_{50} (µg/mL)[2]	RF[3]	Log_{10} EC_{50}	EC_{50} (µg/mL)	RF
Reference	Reference	Wild Type	Wild Type	Wild Type	-1.199	0.063	-	-0.812	0.154	-
RR.35	Rothamsted-UK	Wild Type	N33T & N34T	Wild Type	-1.276	0.053	**0.84**	0.721	5.255	**34.1**
RR.37	Rothamsted-UK	Wild Type	N33T & N34T	Wild Type	-0.342	0.455	**7.18**	-0.260	0.550	**3.56**
RR.58	Rothamsted-UK	Wild Type	I29V &T79N	Wild Type	-0.381	0.416	**6.57**	-0.116	0.766	**4.97**
CHI.47A	Chile	Wild Type	N33T & N34T	Wild Type	-0.517	0.304	**4.80**	-0.146	0.714	**4.63**
CHI.98A	Chile	Wild Type	N33T & N34T	Wild Type	-0.650	0.224	**3.53**	-0.212	0.614	**3.98**
ER.39A	Argentina	Wild Type	Wild Type	Wild Type	-0.501	0.315	**4.98**	-0.124	0.752	**4.87**
ER.53A	Argentina	Wild Type	N33T & N34T	Wild Type	-0.438	0.365	**5.76**	-0.272	0.535	**3.47**
ER.66A	Argentina	Wild Type	N33T & N34T	Wild Type	-1.179	0.066	**1.05**	0.444	2.778	**18.0**
R50.39A	Ruta 50-UY	Wild Type	N33T & N34T	Wild Type	-0.965	0.108	**1.71**	0.175	1.497	**9.70**
R50.44A	Ruta 50-UY	Wild Type	Wild Type	Wild Type	-0.349	0.448	**7.08**	-0.158	0.694	**4.50**
R50.52A	Ruta 50-UY	Wild Type	Wild Type	Wild Type	-0.316	0.483	**7.63**	-0.143	0.720	**4.67**
LE.02B	La Estanzuela-UY	Wild Type	Wild Type	Wild Type	-0.337	0.460	**7.27**	0.064	1.159	**7.51**

Table 3.5 cont.

Isolate	Population	SdhB Genotype	SdhC Genotype	SdhD Genotype	Bixafen			Fluopyram		
					Log_{10} EC_{50}	EC_{50} (µg/mL)	RF	Log_{10} EC_{50}	EC_{50} (µg/mL)	RF
LE.16A	La Estanzuela-UY	Wild Type	N33T & N34T	Wild Type	-0.179	0.663	**10.5**	0.307	2.030	**13.2**
LAN.17A	Michigan-USA	Wild Type	I29V	Wild Type	-0.626	0.236	**3.73**	-0.269	0.538	**3.49**
VAL.02A	Michigan-USA	Wild Type	Wild Type	Wild Type	-1.123	0.075	**1.19**	0.606	4.034	**26.1**
VAL.09A	Michigan-USA	Wild Type	N33T & N34T	Wild Type	-0.563	0.273	**4.32**	-0.292	0.511	**3.31**
ORE.33	Oregon-USA	Wild Type	I29V	Wild Type	-0.497	0.318	**5.03**	1.270	18.62	**121**
ORE.40	Oregon-USA	Wild Type	I29V	Wild Type	-1.409	0.039	**0.62**	1.197	15.75	**102**
ORE.45	Oregon-USA	I44V	I29V	Wild Type	-0.350	0.446	**7.05**	1.102	12.64	**81.9**
ORE.50	Oregon-USA	Wild Type	N33T & N34T	Wild Type	-0.195	0.638	**10.1**	0.924	8.404	**54.5**
SVK.15	Slovakia	Wild Type	F23S & I29V	Wild Type	-0.301	0.500	**7.90**	0.117	1.310	**8.49**
SVK.31	Slovakia	Wild Type	N33T & N34T	Wild Type	-0.330	0.467	**7.38**	0.030	1.070	**6.94**
SVK.37	Slovakia	Wild Type	N33T & N34T	Wild Type	-0.792	0.161	**2.55**	0.593	3.919	**25.4**
CR.05	Czech Republic	Wild Type	N33T	Wild Type	-0.525	0.299	**4.72**	1.177	15.03	**97.4**
CR.25	Czech Republic	Wild Type	Wild Type	Wild Type	-0.404	0.394	**6.23**	0.044	1.108	**7.18**

Table 3.5 cont.

Isolate	Population	SdhB Genotype	SdhC Genotype	SdhD Genotype	Bixafen			Fluopyram		
					Log_{10} EC_{50}	EC_{50} (µg/mL)	RF	Log_{10} EC_{50}	EC_{50} (µg/mL)	RF
CR.32	Czech Republic	Wild Type	Q42E	Wild Type	-0.217	0.607	**9.59**	0.258	1.812	**11.7**
CR.33	Czech Republic	Wild Type	Wild Type	Wild Type	-1.224	0.060	**0.94**	0.599	3.973	**25.7**
AOR.21A	New Zealand	Wild Type	N33T & N34T	Wild Type	-0.960	0.110	**1.73**	0.398	2.500	**16.2**
AOR.33A	New Zealand	Wild Type	N33T & N34T	Wild Type	-0.896	0.127	**2.01**	-0.580	0.263	**1.71**
NT321.17	Reference	Wild Type	Wild Type	Wild Type	0.099	1.255	**19.8**	0.453	2.835	**18.4**

[1]EC_{50} values are the mean of two replicates.
[2]Resistance Factors were calculated by dividing the EC_{50} value of the respective strains by the average EC_{50} value of five SDHI sensitive reference strains.

Table 3.6 Fungicide sensitivity profile of fluopyram resistant field isolates of *Zymoseptoria tritici*.

Isolate	Population	Fluopyram		Isofetamid		Fentin chloride	
		EC_{50}[1] (µg/mL)	RF[2]	EC_{50} (µg/mL)	RF[3]	EC_{50} (µg/mL)	RF[3]
Reference	Reference	0.154	-	0.031	-	0.045	-
RR.35	Rothamsted-UK	5.255	**34.1**	> 50	**> 1609**	0.121	**2.69**
CHI.47A	Chile	0.714	**4.63**	0.166	**5.35**	0.108	**2.41**
ER.66A	Argentina	2.778	**18.0**	20.00	**644**	0.056	**1.24**
R50.39A	Ruta 50-UY	1.497	**9.70**	9.928	**320**	0.031	**0.69**
LE.02B	La Estanzuela-UY	1.159	**7.51**	0.156	**5.03**	0.058	**1.29**
LE.16A	La Estanzuela-UY	2.030	**13.2**	0.532	**17.1**	0.130	**2.89**
VAL.02A	Michigan-USA	4.034	**26.1**	20.00	**644**	0.072	**1.61**
ORE.33	Oregon-USA	18.62	**121**	20.00	**644**	0.108	**2.41**
ORE.35	Oregon-USA	9.821	**63.6**	20.00	**644**	0.076	**1.70**
ORE.38	Oregon-USA	9.822	**63.7**	> 50	**>1 609**	0.137	**3.06**
ORE.40	Oregon-USA	15.75	**102**	> 50	**> 1609**	0.050	**1.11**
ORE.42	Oregon-USA	8.487	**55.0**	> 50	**> 1609**	0.046	**1.03**
ORE.45	Oregon-USA	12.64	**81.9**	> 50	**> 1609**	0.046	**1.03**
ORE.47	Oregon-USA	13.77	**89.2**	> 50	**> 1609**	0.160	**3.57**
ORE.50	Oregon-USA	8.404	**54.5**	20.00	**644**	0.080	**1.79**
ORE.52	Oregon-USA	7.798	**50.5**	20.00	**644**	0.091	**2.03**
SVK.37	Slovakia	3.919	**25.4**	8.000	**257**	0.093	**2.08**
CR.05	Czech Republic	15.03	**97.4**	20.00	**644**	0.124	**2.77**
CR.33	Czech Republic	3.973	**25.7**	20.00	**644**	0.032	**0.70**
AOR.21A	New Zealand	2.500	**16.2**	8.019	**258**	0.040	**0.88**
NT321.17	Reference	2.835	**18.4**	1.429	**46.0**	1.208	**26.9**

[1]EC_{50} values are the mean of two replicates.
[2]Resistance Factors were calculated by dividing the EC_{50} value of the respective strains by the average EC_{50} value of five SDHI sensitive reference strains.
[3]Resistance Factors were calculated by dividing the EC_{50} value of the respective strains by the EC_{50} value of sensitive IPO323 reference strain.

Table 3.7 Frequency (%) of SDHI resistant strains in *Zymoseptoria tritici* field populations. Strains are classified as slightly or highly resistant to bixafen and fluopyram.

Population	n	Bixafen EC_{50} (µg/mL)		Fluopyram EC_{50} (µg/mL)	
		> 0.4[1]	> 4.0[2]	> 1.5[1]	> 5.0[2]
Rothamsted-UK	50	6.0	0.0	16.0	2.00
Chile	71	0.0	0.0	0.0	0.0
Argentina	67	0.0	0.0	1.49	0.0
Ruta 50-UY	53	3.8	0.0	1.88	0.0
La Estanzuela-UY	42	4.8	0.0	2.38	0.0
Michigan-USA	45	0.0	0.0	0.0	2.22
Oregon-USA	57	3.5	0.0	33.3	22.8
Slovakia	39	5.1	0.0	10.2	0.0
Czech Republic	47	2.1	0.0	8.51	2.12
New Zealand	51	0.0	0.0	3.92	0.0

[1]Slightly resistant strains were classified as bixafen > 0.4 µg/mL and fluopyram > 1.5 µg/mL.
[2]Higly resistant strains were classified as bixafen > 4.0 µg/mL and fluopyram > 5.0 µg/mL.

3.3.6 Evolutionary history of *CYP51* variants

A 2029 bp long fragment (Figure 3.1) was sequenced for 102 strains from 10 populations, plus one reference isolate, as described above. Only synonymous mutations and non-protein coding regions were considered in the following analyses, therefore a total of 35 sites were polymorphic when aligned to the reference IPO323 sequence, defining 20 distinct haplotypes due to nucleotide substitutions or deletions that did not produce an amino acid substitution.

Population genetic parameters are listed in Table 3.8 for groups and population comparisons. Table 3.9 shows population differentiation expressed as the coefficient of gene differentiation G_{st} (Nei, 1973). Isolates from South America showed a higher number of polymorphic sites (S = 33) than other groups. Consequently, South America also had slightly higher haplotype diversity and nucleotide diversity than other groups, but the same number of haplotypes as in North America. Within South America, Chile

showed the lowest genetic diversity for all parameters analysed, followed by Argentina. The Uruguayan populations, Ruta-50 and La Estanzuela, had the highest genetic diversity not only in South America but among all populations (Table 3.8). Population differentiation within South America was greatest between Chile and the other populations (Table 3.9).

In North America, Michigan was more genetically diverse than Oregon in all parameters analysed (Table 3.8 and Table 3.9). Meanwhile, in the Eastern European populations, Slovakia had more polymorphic sites, more haplotypes, and greater haplotype diversity than Czech Republic, but the same nucleotide diversity. Interestingly, Michigan was similar to Argentina, La Estanzuela and Ruta-50 while Oregon was highly differentiated from all other populations (Table 3.9).

There was no genetic diversity in New Zealand because only one haplotype was found for synonymous mutations, which was expected since only one CYP51 variant had been found in this population, as presented before (Table 3.8). Hence New Zealand was highly differentiated from all other populations (Table 3.9).

The genealogy of haplotypes based on synonymous mutations in the 2029 bp sequence (gene *CYP51* included) was constructed with the statistical parsimony approach (TCS) implemented in PopArt (Clement *et al.*, 2002). This network showed which isolates share an identical sequence apart from non-synonymous mutations in the *CYP51* coding region. The CYP51 protein variants associated with nucleotide haplotypes are labelled in Figure 3.19.

The reference isolate IPO323 sequence was shown as haplotype number seven (H-7). The network can be divided into two halves in relation to the reference IPO323 (above and below H-7). Only CYP51 variant Y459H appeared in different genetic backgrounds, and the two halves of the network did not share any variants. In one branch were located haplotypes only found in North and South American populations, but none from Chile. The genetic background of these haplotypes was called “North and South American”. These carried primitive amino acid substitutions in the CYP51 sequence, but including some variants in amino acid positions G460 and Y461 previously proved to impact azole sensitivity

(Mullins *et al.*, 2011). Only haplotype H-13, found in North American isolates, had CYP51 variants with I381V and V136A substitutions, and none had both in combination. Isolates in this half of the network did not carry the hypothetically primitive alterations L50S and S188N, nor the double amino acid deletion (ΔY459/G460).

The second half of the network (under H-7 in Figure 3.19), was composed of haplotypes with well-established CYP51 alterations, except for L144F (H-10), which had similar azole sensitivity to the reference. The genetic background of these haplotypes was named "European". In this grouping variants V136A and I381V appeared in combination, sometimes associated with the double amino acid deletion. The S524T was present in different haplotypes. South and North American isolates with either the L50S mutation or double amino acid deletion were placed in this branch of the network.

South American isolates with L50S and the double amino acid deletion (ΔY459/G460) were grouped with European and New Zealand genotypes, showing that these mutations arose in the same genetic background (Figure 3.19; node H-1). In contrast, isolates from Chile and Slovakia with the double deletion but lacking L50S formed a distinct haplotype (H-3), suggesting that the double deletion might have either arisen in or recombined from a different genetic background.

Isolates from Oregon with the genotypes L50S & Y459D were in background H-4, together with isolates from Michigan with genotype L50S & Y461N. However, isolates from Michigan with alteration L50S in combination with S188N were in haplotypes H-8, H-9 and H19 separated from H-4 by at least 12 single nucleotide polymorphisms. Oregon isolates with alterations V136A or I381V had North American genetic background (Figure 3.19; H-13).

There were two common haplotypes with European genetic background, present in all populations. Haplotypes 1 and 4 were separated by 19-point mutations. Some isolates belonging to H-4 carried alterations V136A and I381V with reduced azole sensitivity, but North American isolates with this haplotype did not have either of these alterations. Besides V136A/C and I381V alterations, European

resident H-1 isolates also had the double amino acid deletion and, in some cases, S524T, with the highest RFs for azole fungicides in this study.

The Maximum Likelihood phylogeny of non-recombinant sequences from this dataset was reconstructed in Figure 3.20. The isolate IPO323, highlighted in green, was used as the outgroup, on which the maximum-likelihood tree was rooted. Two main phylogenetic clades emerged from this analysis. The first contained isolates with South and North American genetic background, with presumed primitive CYP51 variants. The other clade was composed of isolates with European genetic background, carrying mutations linked to large reductions in azole sensitivity.

There were two sub-clades in the North and South American clade. Haplotypes from Argentina, Ruta-50 and La Estanzuela, with the South American genetic background formed one, isolates from Oregon and Michigan with North American genetic background formed other. Nevertheless, some of Oregon isolates had either V136A or I381V mutations.

Within the European genetic background, isolates with similar genetic backgrounds carrying resistance-associated mutations in the *CYP51* gene formed tight clusters. A first group did not carry the double amino acid deletion (ΔY459/G460) (Figure 3.20; black). A second group carried the double amino acid deletion with the background mutations S188N & N513K (Figure 3.20; red). South American isolates were present in both clusters of haplotypes with European genetic background, but the North American isolates only appeared in the black cluster.

Table 3.8 Genetic diversity in samples of *Zymoseptoria tritici* field populations.

Group	Population	n[1]	S[2]	h[3]	Hd[4]	K[5]	Pi[6]
Reference	Rothamsted-UK	12	22	6.0	0.8	11	5.3
	Chile	10	13	3.0	0.6	6.3	3.1
	Argentina	10	25	4.0	0.8	9.8	4.8
South America	Ruta 50-UY	10	32	5.0	0.8	11	5.2
	La Estanzuela-UY	10	32	5.0	0.8	8.3	4.1
	Combined	*40*	*33*	*8.0*	*0.8*	*12*	*5.9*
	Michigan-USA	10	24	8.0	0.9	10	4.9
North America	Oregon-USA	10	7	2.0	0.3	2.5	1.2
	Combined	*20*	*24*	*8.0*	*0.7*	*7.3*	*3.6*
	Slovakia	10	23	4.0	0.7	11	5.3
Eastern Europe	Czech Republic	10	19	2.0	0.5	10	5.2
	Combined	*20*	*23*	*4.0*	*0.6*	*10*	*5.1*
New Zealand	New Zealand	10	0	1.0	0.0	0.0	0.0

[1]Number of isolates.
[2]Number of polymorphic sites.
[3]Number of haplotypes.
[4]Haplotype diversity.
[5]Average number of differences.
[6]Nucleotide diversity x 1000.

Table 3.9 Pairwise comparisons of population differentiation (G_{st}) between subsamples of *Zymoseptoria tritici* field populations.

Population[1]	RR	CH	AR	R50	LE	MI	OR	SV	CR	NZ
Rothamsted-UK	*									
Chile	0.044	*								
Argentina	0.009	0.109	*							
Ruta 50-UY	0.002	0.102	-0.019	*						
La Estanzuela-UY	0.034	0.136	-0.025	-0.032	*					
Michigan-USA	0.032	0.118	0.007	0.001	0.007	*				
Oregon-USA	0.234	0.333	0.228	0.219	0.228	0.143	*			
Slovakia	-0.026	0.023	0.015	0.008	0.046	0.051	0.276	*		
Czech Republic	-0.008	0.111	0.016	0.008	0.051	0.066	0.327	-0.027	*	
New Zealand	0.170	0.216	0.333	0.321	0.384	0.363	0.698	0.168	0.285	*

[1]Analyses were carried out with a set of 10 or 12 isolates from the following populations: Rothamsted-UK (RR), Chile (CH), Argentina (AR), Ruta 50-UY (R50), La Estanzuela-UY (LE), Michigan (MI), Oregon (OR), Slovakia (SV), Czech Republic (CR) and New Zealand (NZ).

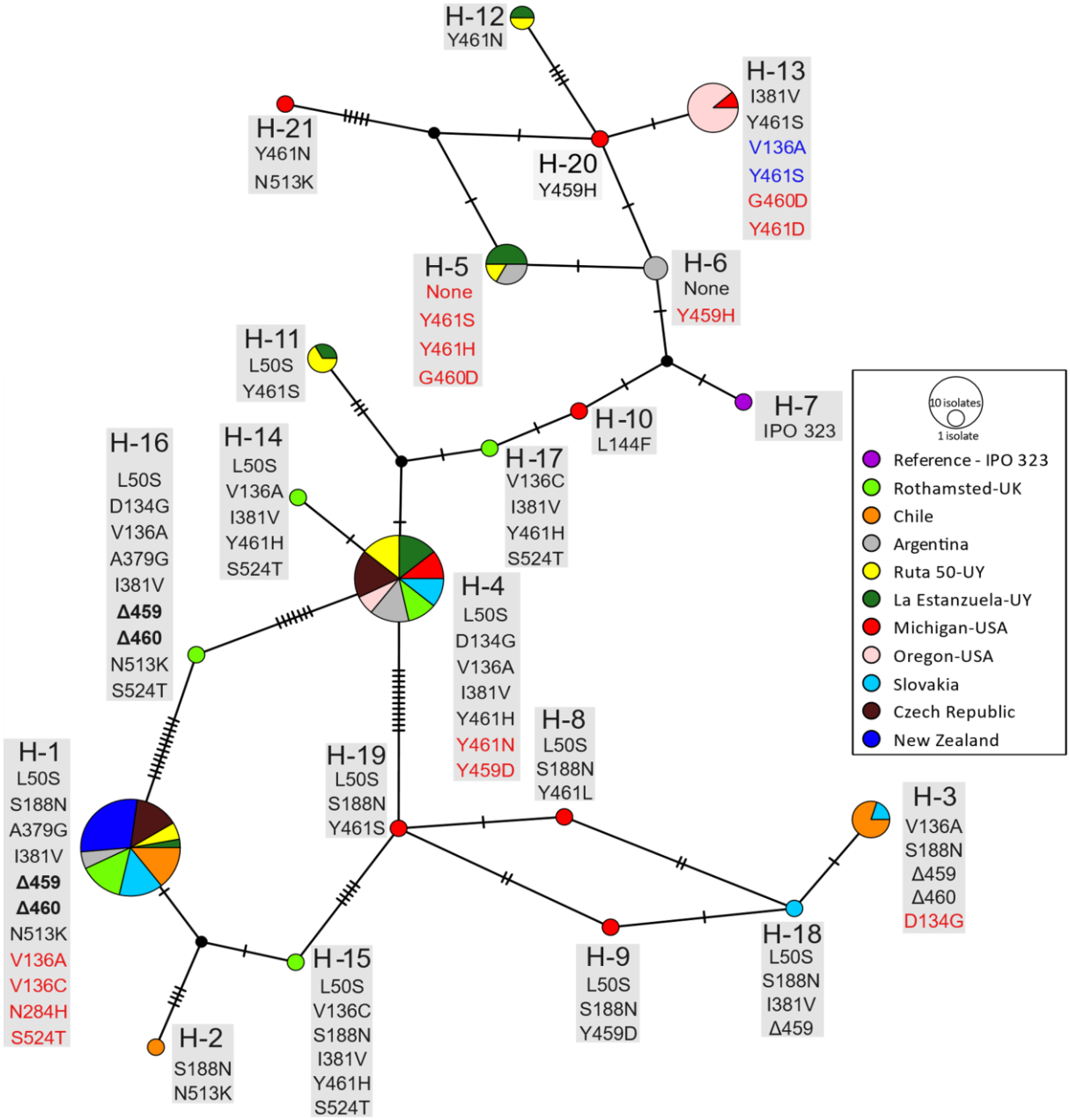

Figure 3.19 Haplotype network constructed with synonymous mutations from a 2029 bp genomic DNA sequence containing the *CYP51* gene (1907 bp) and flanking sequence. Circle size is proportional to haplotype frequency and colours denote the population of origin given in the legend. Small black circles represent hypothetical haplotypes. Black dashes indicate single point mutations. Branch length is not proportional to the distance between haplotypes. CYP51 protein variants associated with each haplotype are listed in the grey boxes. Amino acid substitutions in black or blue form a CYP51 variant, whilst amino acid substitutions in red are found only in some variants.

Figure 3.20 Maximum Likelihood Phylogeny of *Zymoseptoria tritici* field isolates based on synonymous mutations in a 2029 bp genomic DNA sequence containing the CYP51 gene (1907 bp) and flanking sequence. The maximum likelihood tree (ML) was condensed to support values over 70. Branch lengths are not proportional to the number of nucleotide substitutions. CYP51 protein variants associated with each haplotype are highlighted in green (reference isolate IPO323 and CHI 02A), yellow (South American genetic background), blue (North American genetic background), black (European genetic background) and red (European genetic background with ΔY459/G460).

3.4 Discussion

3.4.1 Global distribution of *CYP51* variants and azole sensitivity

The high genetic diversity and sexual reproduction rate in *Z. tritici* contribute, under fungicide selection pressure, towards the evolution of less sensitive strains within a population. With the widespread use of single-site fungicides to control *Z. tritici*, the molecular mechanisms causing resistance are usually in the gene(s) for the target site of the fungicide molecules. Azole resistance evolution has developed more gradually than for some other single-site fungicides and does not result in complete loss of sensitivity or complete cross-resistance between all fungicides in the azole class. As an example, only small erosion of azole effectiveness against STB has been reported in *Z. tritici* populations from North America and New Zealand, sampled in the same regions as those in this thesis, whereas abrupt loss of sensitivity to QoI fungicides occurred in the same populations (Estep *et al.*, 2013, 2015; Stewart *et al.*, 2014; Hayes *et al.*, 2016; Hagerty *et al.*, 2017). In the case of azole fungicides, various molecular mechanisms are associated with resistance in *Z. tritici* (Leroux & Walker, 2011; Cools & Fraaije, 2013; Kildea *et al.*, 2019), thus the accumulation of molecular modifications in strains of this fungus can result in high loss of sensitivity towards this class of fungicide.

The effect of different amino acid alterations in azole sensitivity has already been demonstrated in *Z. tritici* by heterologous expression (Cools *et al.*, 2010, 2011) and molecular modelling (Mullins *et al.*, 2011) of the target site protein, CYP51. The effects on azole sensitivity of combinations of these alterations have also been investigated using regression analysis (Dooley, 2015). Therefore, the present work attempted to determine the CYP51 amino acid alterations linked to different azole sensitivities in populations of *Z. tritici* worldwide.

In general, sensitivity decreased with an increased number of amino acid alterations in the CYP51 variants. Isolates from South America displayed a wide range of CYP51 variants that can be distinguished in two profiles: Chile, sampled in a region in the west side of the Andes mountains, with a characteristic Oceanic climate - Cfb; and the other populations from South America (from Uruguay

and Argentina), sampled East of the Andes, in a region of Humid Subtropical climate - Cfa (Kottek *et al.*, 2006).

Chilean isolates grouped in four clusters distinguished by tebuconazole and prochloraz sensitivities. One cluster is sensitive to both fungicides, represented by the CYP51 variant S188N & N513K. These amino acid alterations have been reported as not affecting azole sensitivity, but rather being part of the pre-fungicide variation in the protein, along with other mutations L50S (Leroux *et al.*, 2007; Mullins *et al.*, 2011). Mullins *et al.* (2011) showed that S188N & N513K alterations were located exposed to the outside of the folded protein and therefore far from the binding pocket of azole molecules.

Another cluster showed positive cross-resistance between tebuconazole and prochloraz. A CYP51 variant found in this cluster was L50S, S188N, Δ459/460 & N513K. As with S188N and N513K above, L50S is not thought to affect DMI sensitivity, because its location is away from the docking pocket of any azole (Mullins *et al.*, 2011). Thus, the double amino acid deletion (ΔY459/G460) present in this variant is responsible for fungicide resistance. Leroux *et al.* (2007) found DMI resistant strains with the double deletion ΔY459/G460 always associated with mutations S188N & N513K, suggesting that the deletion happened in a haplotype(s) containing those variants. The deletion is associated with a large increase in the cavity volume of CYP51, consequently reducing proximity between protein residues and azole molecules. This is especially marked in CYP51 variants simultaneously carrying L50S, S188N & N513K. Because the distance between residue I381 and prochloraz is less altered, resistance to this compound is lower than for tebuconazole (Mullins *et al.*, 2011).

Two other clusters had contrasting tebuconazole and prochloraz sensitivity. In one, strains were moderately resistant to prochloraz and epoxiconazole but sensitive to tebuconazole, due to the triple CYP51 variant V136A, S188N & Δ459/460. Alteration V136A is known to cause resistance to prochloraz because of conformational change in the enzyme binding pocket. As above, the deletion reduces proximity between CYP51 residues and other azoles, explaining epoxiconazole resistance. However, the conserved residue K148 remains close to tebuconazole, explaining the high sensitivity to this

molecule (Mullins *et al.*, 2011; Cools *et al.*, 2013). The last cluster had CYP51 variant L50S, S188N, A379G, I381V, Δ459/460 & N513K. According to Mullins *et al.* (2011), the single amino acid substitution I381V probably reduces the cavity volume of the protein. This, combined with the deletion at positions 459/460, reduces the interaction of the catalytic residues with epoxiconazole and tebuconazole. It, therefore, explains the higher RFs to these two compounds, as also observed by Stammler *et al.* (2008).

Populations from Argentina, Ruta 50 and La Estanzuela had similar CYP51 variants among them, which can be explained by gene flow of resistant alleles due to the movement of ascospores between sites in a contiguous wheat-sown region. Ruta 50 and La Estanzuela were sampled only 6 km apart, while Argentina was located around 170 km from these. Differences in azole sensitivity between these populations, addressed in the previous chapter, must be due to different frequency of the CYP51 variants in each population.

Single amino acid substitutions were present in Argentina, Ruta 50 and La Estanzuela. These single amino acid substitutions at positions 459, 460 and 461 of CYP51 are known to increase the enzyme cavity volume and fungicide resistance is caused by reallocation of specific residues further from azoles (Mullins *et al.*, 2011). In this study, we have observed a reduction in sensitivity to prochloraz and a more pronounced reduction in sensitivity to tebuconazole in the presence of the single amino acid substitutions in the positions mentioned above. Nonetheless, no sensitivity change was observed regarding epoxiconazole. Higher tebuconazole and prochloraz resistance factors were detected in isolates with changes at Y461, in agreement with Mullins *et al.* (2011) who predicted that the loss of that tyrosine residue caused a higher impact on azole binding to the enzyme pocket. Amino acid alterations at positions 459 and 461 increased resistance to epoxiconazole in *S. cerevisiae* transformants heterologously expressing *Z. tritici* CYP51 (Cools *et al.*, 2010) and in *Z. tritici* field isolates (Cools *et al.*, 2011). This study did not find evidence of epoxiconazole resistance in isolates carrying amino acid alterations between positions 459 and 461, either as single substitutions or variants alongside alterations L50S and S188N. This finding includes isolates from South America, Michigan, and

Slovakia when compared to the azole sensitive reference isolates. The only exceptions are two field isolates from Oregon-USA with CYP51 variant L50S & Y459D expressing an epoxiconazole RF of 8.5. Isolates with "wild type" CYP51 found in South America had an RF of 0.3 to epoxiconazole and tebuconazole and 0.1 to prochloraz, compared to the six azole sensitive reference strains. Reduced fungal growth in media is a possible explanation for non-European isolates showing a slightly different azole sensitivity in comparison to what has been demonstrated in previous studies with "wild type" *Z. tritici* strains.

The populations from Argentina, Ruta 50 and La Estanzuela had in common the CYP51 variant L50S, I381V & Y461H. It was the only haplotype in these populations with reduced sensitivity to all three azoles tested, with RF much higher to tebuconazole than to prochloraz. Field isolates with this variant were common in Europe in 2007 (Stammler *et al.*, 2008). The CYP51 enzyme with these alterations is known to have a smaller fungicide-binding cavity than the wild type, mostly due to the incorporation of alteration I381V (Mullins *et al.*, 2011). In each of these three populations, most of the non-genotyped isolates were in a sensitivity phenotype cluster with this variant, presumably because it has been under selection in recent years. Conversely, this CYP51 variant was not present in Chile. This suggests either that recent azole fungicide use has been rather different between these populations, for which insufficient records are available in the literature, or that the variant has not yet arisen by gene flow from Europe or by local mutational events in Chile. However, the I381V alteration was present in CYP51 variants in all four populations from South America.

There was only one CYP51 variant in common to all South American populations. This was L50S, S188N, A379G, I381V, Δ459/460 & N513K. Although expressing the expected phenotype towards azoles as described by Mullins *et al.* (2011), some Chilean isolates with this variant had significantly reduced sensitivity compared to others. This CYP51 variant was found in Ireland with and without an insert of ~800 bp in the promoter region of the *CYP51* gene; the presence of the insert was associated with a significant reduction of azole sensitivity (Dooley, 2015). The approximately 800 bp insert was detected

in three strains from Chile with higher RFs, whilst isolates with the same CYP51 haplotype in the other populations from South America carried no inserts. Analysis of the PCR amplicon in one strain from Chile revealed that this insert was in fact 862 bp long. Kildea *et al.* (2019) have recently described an 862 bp insert in European strains of *Z. tritici* to be associated with CYP51 haplotypes carrying the double deletion (Δ459/460). This insert could be responsible for *CYP51* overexpression and consequently increased azole resistance in the same way as the 120 bp insert described by Cools *et al.* (2012). Increased levels of azole resistance have been shown before in *Z. tritici* strains carrying promoter inserts (Chassot *et al.*, 2008; Leroux & Walker, 2011). However, two other studies have not shown similar insertions to cause an increase in gene expression (Omrane *et al.*, 2015; Kildea *et al.*, 2019).

In New Zealand, a single CYP51 variant L50S, S188N, A379G, I381V, Δ459/460 & N513K was found in all 10 isolates that were sequenced. The highly prochloraz sensitive phenotype is explained by the absence of amino acid changes at position 136 and the presence of both A379G and I381V. Seven of the isolates had a large insert in the *CYP51* promoter and expressed higher azole RFs. One of these inserts was sequenced and was 787 bp long. This size of the insert has not previously been reported. It was in the same position as the 862 bp insert previously described. Gradual epoxiconazole sensitivity decrease has already been reported in the lower region of the north island of New Zealand (Stewart *et al.*, 2014), the region from which the samples in the current study came. The variants found in this study could also explain the poor field trial performance of this molecule in the Canterbury region of New Zealand (FAR, 2018), located in the South Island, which would mean that *Z. tritici* isolates carrying CYP51 genotypes with resistance to tebuconazole might be widespread in New Zealand. In fact, this thesis included field isolates sampled in the South Island (data not shown). Those from Canterbury had a similar phenotype to the population from the North Island. However, those from Otago, located in the south part of South Island, had six out of twelve isolates highly sensitive to tebuconazole but highly resistant to prochloraz, suggesting they carry CYP51 variants with substitutions at codon 136. Thus, we hypothesize that these populations from New Zealand are independently evolving resistance to azoles, either by *de novo* mutations or independent migration events of European isolates.

North American populations showed on average similar azole sensitivity among them, except with epoxiconazole for which resistance levels were higher in the Oregon population, but CYP51 variants were rather different between sites. Amino acid substitutions responsible for azole loss of sensitivity in Michigan were all located at codon positions 459 and 461. These were either single alterations or appeared in combination with L50S, S188N or N513K. As explained above, these isolates did not show reduced epoxiconazole sensitivity, and the Michigan population was more sensitive than Oregon to this compound. It seems likely that all, except one, isolates from Michigan carry alterations at 459 or 461 positions because they clustered together and showed a strong correlation between tebuconazole and prochloraz sensitivity.

The Oregon collection had only four different CYP51 haplotypes, that grouped into three clusters. Those with alterations only at codons 459 or 460 showed cross-resistance to tebuconazole and prochloraz. The other variants were I381V & Y461S and V136A & Y461S. The effects of amino acid changes at positions 381 and 136 were explored above and explain the contrasting pattern of tebuconazole and prochloraz sensitivity between these clusters. The presence of such genotypes is presumably due to higher disease pressure and greater use of fungicide in Oregon than in Michigan.

The *Z. tritici* isolates sampled in Oregon show stepwise evolution of resistant mutations. Isolates sampled in 1990 (Zhan *et al.*, 2006) and 1992 (Estep *et al.*, 2015), during the early stages of azole fungicide adoption in Oregon (Hagerty *et al.*, 2017), did not show any decrease in azole sensitivity or CYP51 amino acid substitutions linked to azole resistance. By 2012, after a substantial increase in the use of azoles in the mid-2000s (Hagerty *et al.*, 2017), sampled isolates had amino acid changes at codons Y459, G460, and Y461 (Estep *et al.*, 2015). Changes in these amino acids, giving moderate increases in resistance, are required to accommodate variants conferring larger changes in azole sensitivity, like I381V and V136A, that otherwise would be lethal (Cools *et al*., 2010; Cools & Fraaije, 2013). There are no reports of prochloraz use in wheat crops in Oregon, so the reason for the emergence of CYP51 variants with the V136A alteration, but without prochloraz selection pressure,

remains unclear. A possible next step in azole resistance evolution in Oregon is the deletion of two amino acids (ΔY459/G460), characteristic of modern European CYP51 variants with increased resistance to azoles (Stammler *et al.*, 2008). With the recent adoption of prothioconazole in the Willamette Valley (Sykes *et al.*, 2018), the mutations S524T or D134G might be favoured as well.

A diverse set of CYP51 variants was found in European *Z. tritici* field populations genotyped in this study. In Rothamsted, UK, none of the isolates was fully-sensitive to any of the azoles tested. Amino acid change I381V, responsible for reduced azole sensitivity (Cools *et al.*, 2010), was present in all CYP51 variants found in Rothamsted, presumably because it has been positively selected by recent fungicide spraying programs (Dooley, 2015). Moreover, only two isolates did not have a substitution at codon 136, linked to prochloraz resistance, alongside I381V.

Six out of nine haplotypes found in Rothamsted, and one haplotype from Slovakia, had the alteration S524T responsible for decreased sensitivity to azoles, particularly prothioconazole (Cools *et al.*, 2011; Cools & Fraaije, 2013). This substitution changes CYP51 conformation, further reducing the interaction of fungicides with the binding cavity (Cools *et al.*, 2011). In the present study, S524T was found in isolates with the higher RFs to epoxiconazole. Alteration S524T was reported, in combination with Y137F, in Europe (Cools *et al.*, 2011; Cools & Fraaije, 2013) and North America (Estep *et al.*, 2015) before wide adoption of prothioconazole by wheat growers. This haplotype, Y137F & S524T, conferred a decrease in azole sensitivity and an increase in enzyme activity in comparison to the early 1990s haplotype with Y137F alone (Cools *et al.*, 2011). In modern CYP51 variants, the introduction of S524T, in a genetic background that already had multiple other alterations including Δ459-460, I381V, and V136A, further reduced *in vitro* sensitivity to the azole fungicides prothioconazole and epoxiconazole (Cools *et al.*, 2011), as observed in this study, demonstrating the stepwise evolution of resistance. Another alteration only found in Europe was D134G, reported since 2008 in combination with I381V and V136A (Stammler *et al.*, 2008) and now present in various CYP51 variants.

As discussed in the previous chapter, Eastern European populations were more sensitive to epoxiconazole and prochloraz than the NW-European population from Rothamsted. This is explained by the CYP51 variants present. Modern CYP51 variants associated with high levels of azole resistance have emerged in NW-European wheat-growing countries because fungicide selection pressure is higher, and fungicides have been used for longer than anywhere else. It is proposed that isolates migrate eastwards with the movement of ascospores along with the prevailing European wind directions (Brunner *et al.*, 2008). There were some substantial differences in CYP51 haplotype composition between Rothamsted and the Eastern European samples. Not all isolates found in Eastern Europe were resistant to all three azoles. It is possible that the frequency of the least sensitive variants is lower in populations under less selection pressure because of slight fitness costs of the most fungicide resistant mutations.

Although wheat crop production in Slovakia and Czech Republic does not rely on the same levels of intense use of fungicides as in Western Europe, some of the CYP51 variants found in Eastern European populations are more evolved towards azole resistance than those seen outside of Europe. The double deletion of amino acids Y459 and G460 were observed in most isolates. Both populations had at least one CYP51 variant with the alteration I381V alongside V136A, not found outside Europe. Additionally, the amino acid alteration S524T was found in a modern CYP51 variant from Slovakia with low sensitivity to azoles, particularly epoxiconazole.

In both Rothamsted and the Czech Republic, all isolates with the variant L50S, S188N, I381V, Δ459, Δ460 & N513K carried a 120 bp insert in the promoter region of the CYP51 encoding gene, as described by Cools *et al.* (2012) and present in the UK isolates since 2009. *Z. tritici* isolates carrying the 120 bp promoter insert described by Cools *et al.* (2012) had between 10 and 40-fold higher levels of constitutive *CYP51* transcript and between 7 and 16-fold increase in EC_{50} values of all azoles tested. In the present study, all isolates with this amino acid variant carried this promoter insert, so it is not possible to assess the relative contribution to the resistance of the promoter insert compared to the

coding changes. The isolate L50S, S188N, I381V, Δ459, Δ460 & N513K with 120 bp insert from Czech Republic had RFs 1.4-fold higher to epoxiconazole and tebuconazole and 2.5-folds higher to prochloraz than similar isolates from Rothamsted. This isolate from Czech Republic also showed an MDR phenotype, resistant to 10 µg/mL of tolnaftate. Sequencing of the MFS1 efflux pump promoter showed a 267 bp insert, not reported in the literature. The new insert is in the same position as the 339 bp insert described by Omrane *et al.* (2017). Interestingly, such an insert was also found in one isolate from Rothamsted with the MDR phenotype. These MDR strains had enhanced resistance of 8 and 5-fold to bixafen and fentin chloride, respectively, in comparison to IPO323. Although these RFs are much lower than those found in MDR strains carrying the 519 bp (Type I) insert (Omrane *et al.*, 2015), we hypothesize that the 267 bp insert is also correlated to efflux pump overexpression. Another isolate from Rothamsted with the MDR phenotype carried the 339 bp insert. Omrane *et al.* (2017) reported strains with this genotype to have similar levels of fungicide resistance to strains with the 519 bp insert. That contradicts the findings in the present study, for which RFs in the strain with the 339 bp insert were lower than those associated with the 519 bp insert. Although overexpressing the MFS1 transporter, expression levels in strains with 339 bp insert were significantly lower than those in strains with 519 bp insert (Omrane *et al.*, 2017). Interestingly, two isolates from Oregon, USA, grew at 10 ppm of tolnaftate, but the MFS1 promoter showed a similar sequence to that in IPO323. Other efflux pumps, such as additional MFS transporters or ATP-binding cassette (ABC) transporters, may be associated with the phenotype seen in these isolates (Zwiers *et al.*, 2003; Stergiopoulos *et al.*, 2003; de Waard *et al.*, 2006; Leroux & Walker, 2011).

3.4.2 QoI and MBC resistance

Unlike azoles, the emergence of resistance to MBC and QoI fungicides in *Z. tritici* is due to single mutations causing a qualitative change, characterized by a bimodal sensitivity distribution in the population. QoI fungicides have been used to control Septoria tritici blotch since 1996, but resistance emerged and rapidly spread in Europe (Fraaije *et al.*, 2005). QoIs inhibit a target site, cytochrome bc1, encoded by the mitochondrial gene *cyt b* (Gisi *et al.*, 2002). The substitution of glycine by alanine at

position 143 (G143A) in cytochrome bc1 interferes with the linker part of the QoI fungicide molecule, reducing its interaction with the binding pocket and conferring high resistance factors. The substitution of phenyl-alanine by leucine at position 129 (F129L) interferes with the toxophore binding of QoI molecules, conferring a lower resistance factor than G143A (Sierotzki, 2015), but has still emerged in some pathogens. In the present study molecular and fungicide sensitivity tests showed that strains, from all populations, with azoxystrobin EC_{50} value > 1.0 µg/mL carried G143A, in accordance with previous publications (Fraaije *et al.*, 2005; Sierotzki, 2015). Strains with EC_{50} value < 0.5 µg/mL had the wild type *cyt b*. The F129L mutation was only found in two isolates from New Zealand with intermediate sensitivity. In European populations of *Z. tritici* G143A quickly became dominant, outcompeting F129L (Lucas & Fraaije, 2007; Kildea *et al.*, 2010).

The G143A resistant allele is known to have emerged independently in different genetic backgrounds and spread in European countries from west to east by airborne ascospores (Fraaije *et al.*, 2005; Torriani *et al.*, 2009). Nonetheless, resistance emerged less quickly in Southern and Eastern Europe (Lucas *et al.*, 2015; Sierotzki, 2015).

The first signs of QoI resistance in Czech Republic were reported in 2002 (Tvaružek *et al.*, 2012). In the population sampled in 2016, we have found 83% resistant strains in the Czech Republic. In contrast, most of the sample from Slovakia was sensitive to azoxystrobin, with only 10% of isolates potentially carrying G143A. Although the difference in frequency of resistant alleles in two neighbouring countries can be associated with differences in QoI usage, gene flow would play a major role in bringing G143A into Slovakia. However, we can only speculate about what regional particularities in Slovakia led to non-fixation of this allele. It could be the result of low migration of windborne ascospores into the region where the Slovakian population was sampled due to natural barriers. This would be in accordance with Drabešová *et al.* (2013), for which asymmetrical gene flow was found among *Z. tritici* populations from Czech Republic. Higher influx of migrants in Kroměříž-CR due to the location of a cereal breeding station (Drabešová *et al.*, 2013), and consequently movement of infected material,

could also be the reason why a higher frequency of G143A was found in this location in comparison to Slovakia.

Benzimidazoles block nuclear division by binding to β-tubulin and preventing microtubule assembly (Davidse & Ishii, 1995; Young, 2015). MBC resistance in *Z. tritici* is caused by a single point mutation in the β-tubulin encoding gene leading to amino acid substitution E198A, resulting in very high cross-resistance to different MBC compounds (Griffin & Fisher, 1985). In the present study, it was demonstrated that the E198A allele is present in isolates able to grow at 1.0 µg/mL of carbendazim in all populations. This mutation is now widespread in European populations at high frequencies (Lucas *et al.*, 2015). In contrast to QoI mutation G143A, frequencies of the MBC resistance allele E198A are similar between Eastern European populations, varying from 100% in Czech Republic to 74% in Slovakia, and at high frequency in Rothamsted (94%). There are not many published works about fungicide resistance in *Z. tritici* populations from Eastern Europe, but benzimidazole resistance was at high frequency in Polish isolates sampled in 2012-13 (Pieczul & Świerczyńska, 2015).

QoI and MBC resistance in *Z. tritici* had not previously been explored in South American populations. The present study has shown that both are present and widespread in populations from different countries, and the molecular mechanisms responsible are the known amino acid substitutions G143A in cytochrome bc1 and E198A in β-tubulin. The high frequencies of G143A in *Z. tritici* populations sampled east of the Andes mountains (Argentina, Ruta 50 and La Estanzuela) and low frequency in Chile, located westward of the Andes, corroborates the results found for CYP51 variants. The geographical isolation of Chile in relation to other populations from South America prevents movements of airborne ascospores. Further investigation of the mitochondrial genome can show if the G143A mutation has in fact evolved independently in different South American populations of *Z. tritici* as it has in Europe (Torriani *et al.*, 2009). Faster evolution of QoI resistant alleles eastward of the Andes might be associated with higher use of strobilurin fungicides. In contrast to G143A, the MBC resistant allele E198A was found at low frequency (26%) in La Estanzuela, even though the neighbour

populations of Ruta 50 had a much higher frequency (77%). This might be the result of an adaptation of local populations to recent MBC applications in Ruta 50.

In North America, the MBC resistant allele E198A was only observed in Oregon, at low frequency (23%). In contrast, G143A was also present in Michigan. The QoI resistant allele was first reported in Oregon in isolates sampled in 2012 (Estep *et al.*, 2013). Estep *et al.* (2015) found G143A alleles at 10% and 83% of the strains in two populations sampled approximately 100 km apart in the Willamette Valley, western Oregon. Moreover, G143A mutations in Oregon isolates of *Z. tritici* are thought to have emerged in several independent *de novo* mutation events (Estep *et al.*, 2015). The present study is consistent with previous surveys which show that the G143A allele is nearly-fixed in a wide region of the Willamette Valley (Hagerty *et al.*, 2017). Our population sampled in 2016 showed 68% of G143A alleles in the same location where Hagerty *et al.* (2017) found only 22% of resistance in 2015, even though both populations were collected in a region isolated from commercial wheat fields and not subject to intense fungicide selection pressure.

After observations of QoI field failure in controlling populations of *Z. tritici* in New Zealand, isolates from the lower region of North Island were assessed and exhibited azoxystrobin EC_{50} values over 100-fold higher than the sensitive references (Stewart *et al.*, 2014). Although these authors did not characterize the molecular mechanism responsible for QoI resistance, their findings are in accordance with the 83% of G143A alleles found in this research. Moreover, in the present study, two isolates from New Zealand had the F129L mutation, with much lower azoxystrobin RFs (< 50-folds) in comparison to those associated with G143A.

3.4.3 SDHI resistance

The SDHI fungicides inhibit complex II of fungal respiration, the succinate dehydrogenase. SDHI fungicides bind to the ubiquinone-binding site, formed by residues of the subunits SdhB, SdhC and SdhD (Stammler *et al.*, 2015). Mutants of *Z. tritici* resistant to SDHIs carried amino acid substitutions in one or more of these subunits (Fraaije *et al.*, 2012; Dooley *et al.*, 2016a; Kirikyali *et al.*, 2017; Rehfus *et*

al., 2018; FRAC, 2019). The present study measured sensitivity to two SDHI fungicides, bixafen and fluopyram, as previous studies had shown some differences in cross-resistance between those compounds. A selection of *Z. tritici* isolates from all regions sampled had the Sdh subunits sequenced to check for point mutations. Isolates whose Sdh subunits were sequenced had the highest EC_{50} values for bixafen within each population, although most were still within the sensitive range for this SDHI (RF < 10). Only two isolates were classified as moderately sensitive, with RFs just slight above 10, and both carried only the background mutations C-N33T & N34T, not thought to be linked to insensitivity. No point mutations were found in the SdhD subunit. I44V was found in SdhB, in an isolate from Oregon sensitive to bixafen.

Although different mutations were found in SdhC they all appeared to be unconnected to changes in bixafen sensitivity. Strains from South America and New Zealand had either a wild type sequence in SdhC or the isoform C-N33T & N34T, which has previously been reported in *Z. tritici* European field isolates showing similar SDHI sensitivity to the wild type (Fraaije *et al.*, 2012; Dooley *et al.*, 2016a; Kirikyali *et al.*, 2017). In North America, the alteration C-I29V also appeared in some isolates. Eastern European populations showed amino acid substitutions C-F23S & I29V and C-Q42E. The first two have already been found as background mutations in European isolates, while the last one is at the same residue as the already known C-Q42P substitution, all with no impact on SDHI sensitivity (Fraaije *et al.*, 2012; Dooley *et al.*, 2016a). One single isolate from Rothamsted had the genotype C-I29V & T79N. Although the present study found this amino acid exchange was associated with a sensitive phenotype for both SDHIs tested, the single amino acid alteration C-T79N has been confirmed to cause a low level of SDHI resistance in European isolates (Dooley *et al.*, 2016a). In addition, it was one of the most frequent haplotypes found in 2016 and showed a partial decrease in SDHI efficiency for *in planta* STB control, to bixafen only (Rehfus *et al.*, 2018). *Z. tritici* European field isolates have been reported carrying different Sdh mutations with high levels of SDHI resistance (Dooley *et al.*, 2016a; Kirikyali *et al.*, 2017; Rehfus *et al.*, 2018; FRAC, 2019), but there are no reports of resistance to this fungicide class in *Z. tritici* populations in Czech Republic, Slovakia or from other continents.

Fluopyram binds to the ubiquinone-binding site of Sdh in a different way than bixafen, which explains the lack of cross-resistance between these SDHIs (Fraaije *et al.*, 2012; Yamashita & Fraaije, 2018). In fact, the present study has found isolates with both moderately sensitive and insensitive phenotypes to fluopyram, not correlated to the amino acid alterations in Sdh subunits discussed above. Altered expression of efflux pumps could be responsible for reduced sensitivity. Nevertheless, none of the fluopyram resistant strains showed an MDR phenotype with resistance to the efflux pump substrate fentin chloride, and only two isolates from Oregon were resistant to tolnaftate. Moreover, most strains were cross-resistant between fluopyram and isofetamid, another SDHI. Yamashita & Fraaije (2018) have shown cross-resistance between fluopyram and isofetamid in strains with no key alterations in Sdh subunits forming the ubiquinone-binding pocket. Furthermore, these authors also showed cross-resistance to this pair of fungicides in mitochondrial extracts of resistant strains. Because strains sampled before the widespread use of SDHIs showed high cross-resistance to the above-mentioned compounds, it is thought that this non-target mechanism of resistance stands as background genetic variation (Yamashita & Fraaije, 2018).

In a recent publication, Steinhauer *et al.* (2019) reported on a dispensable paralogue of *SdhC* in *Z. tritici*. They showed that this gene has differential levels of expression and alternative splicing of mRNA in different isolates. This paralogue, in addition to the competition arising between the two SdhC proteins for inclusion in the succinate dehydrogenase complex, may modulate cross-resistance to fluopyram and isofetamid. The isoleucine at position 78 of the SdhC subunit encoded by the paralogous *SdhC* gene is likely to be the key residue that causes the interaction of these two specific SDHI compounds with the ubiquinone-binding site (Steinhauer *et al.*, 2019). The paralog was present in 18% of a *Z. tritici* population from Oregon-USA collected in 1990. This mechanism of resistance was therefore present in the standing gene pool more than 20 years before the introduction of SDHI fungicides in that region, which is consistent with the high frequency of fluopyram-resistant isolates in the Oregon population sampled in 2016 in the present study.

3.4.4 Emergence and evolution of resistance to azole fungicides

Finding that CYP51 amino acid changes leading to azole resistance were present in South and North American populations of *Z. tritici* raised the question of whether mutations that confer these alterations arose independently in different genetic backgrounds (homoplasy) or they arose once in Europe and then spread to other continents. To understand how CYP51 alterations emerged in South and North American populations of *Z. tritici* the *CYP51* genetic background of 10 strains per population was analysed and compared to European *CYP51* sequences.

The differences in diversity levels of *CYP51* observed between some populations probably reflect different evolutionary histories. Thus, we can affirm that Chile has evolved CYP51 variants in a different genetic background from the three South American populations on the other side of the Andes. Higher fungicide usage in Chile can be associated with selective sweeps, which explains the lower genetic diversity in comparison to Argentina, Ruta-50 and La Estanzuela. Moreover, moderate genetic differentiation was observed between CYP51 haplotypes from Chile and the three other South American populations. None of the CYP51 haplotypes from Chile had the South American genetic background. However, some strains from Chile had the same CYP51 haplotype with European genetic background found in Argentina and Uruguay. This haplotype carried the amino acid alterations L50S and the double amino acid deletion (ΔY459/G460). Other strains from Chile that carried the double deletion but not L50S had the same haplotype seen in strains sampled in Slovakia. This is evidence that the current *Z. tritici* CYP51 variants sampled in Chile descend from two different European lineages and are not related to the South American genetic background.

Another point to be made is that the double amino acid deletion (ΔY459/G460) seems to have recombined in two distinct genetic backgrounds in Europe. The haplotype network showed these two genetic backgrounds associated with the deletion to be separated by many single point mutations. However, it would need a haplotype network constructed with more historical European isolates to conclude this observation. Moreover, the phylogenetic reconstruction of non-recombinant haplotypes

indicated that the double amino acid deletion (ΔY459/G460) present in different CYP51 variants shared a common ancestral. Previous studies have concluded that the double amino acid deletion present in modern European *Z. tritici* strains emerged in only one ancestral haplotype (Brunner *et al.*, 2008). In contrast, mutations V136A and I381V were reported to have emerged in different ancestral lineages (Leroux *et al.*, 2007; Brunner *et al.*, 2008; Dooley, 2015), in agreement with findings from this study.

Strains from Oregon with reduced azole sensitivity had amino acid changes V136A and I381V with the North American genetic background, shown not to be related to the European genetic background containing these same mutations in both the haplotype network and the phylogenetic analysis. Moreover, only one haplotype from Oregon with the L50S alteration had the European genetic background. Meanwhile, just two haplotypes from Michigan had the North American genetic background, whilst all others showed the European genetic background. This is strong evidence that these two North American populations have different origins and there is no gene flow between then. Indeed, pairwise comparisons of *CYP51* genetic background also showed Michigan to be more closely related to European populations, and South American populations from Argentina and Uruguay, than to that from Oregon. Furthermore, Oregon is evolving azole resistance in parallel to European populations, as it has some of the CYP51 alterations (V136A and I381V) found in European strains in the early 2000s (Cools & Fraaije, 2013). Azole selection pressure in Oregon is selecting few adapted CYP51 variants according to the low haplotype diversity seen in this population. This is supported by the higher importance of STB in the Willamette Valley of Oregon in comparison with other North American regions since, in the first, fungicide use to control this disease has been intense and loss of effectiveness of azoles has been extensively reported (Estep *et al.*, 2015; Hayes *et al.*, 2016; Hagerty *et al.*, 2017; Sykes *et al.*, 2018). In Eastern European populations, all CYP51 haplotypes had European genetic background, just displaying the already known selective replacement of old European haplotypes as highly-resistant *CYP51* alleles that emerged in NW-Europe move eastwards through wind-dispersed ascospores (Brunner *et al.*, 2008).

Finally, New Zealand had only one haplotype, with European genetic background, the same one common between all South American populations. Interestingly, this haplotype (with CYP51 alterations L50S, S188N, A379G, I381V, Δ459/460 & N513K) was found in all populations except North America. Moreover, this CYP51 variant was always found with the European genetic background, although strains differed in the presence or absence of promoter inserts. This is evidence that European *Z. tritici* strains carrying a modern azole-resistant CYP51 variant have migrated into other continents. Conversely, McDonald *et al.* (2019) concluded that *de novo* mutations, rather than migration of European isolates, were responsible for parallel evolution of the CYP51 variant L50S, S188N, A379G, I381V, Δ459/460 & N513K in Tasmanian isolates of *Z. tritici*. However, the same authors found that the *CYP51* sequences in Tasmanian isolates were identical to European isolates' partial *CYP51* sequences at both non-synonymous and synonymous level, what under the scope of the present thesis means that they had the same CYP51 genetic background.

Considering this analysis, it is proposed that mutations conferring azole resistance in South America have few different origins. Haplotypes with the South American genetic background, located east of the Andes (Argentina and Uruguay), are displaying signs of parallel evolution to the historical European *CYP51* evolutionary pathway. As an example, amino acid changes in CYP51 positions Y459-Y461 were found, shifting the sensitivity of some azoles, in Argentina and Uruguay. However, these same populations also had modern CYP51 variants causing a higher reduction in azole sensitivity, and these are proposed to have emerged in Europe and then migrated into South America. In Chile, an isolated population in relation to the three others in South America, resistant CYP51 variants seem to have emerged from two different European lineages that migrated into Chile.

A central question arising from the data in this chapter is whether European strains carrying modern CYP51 variants associated with resistance against azole fungicides are migrating to distant regions, such as South America and New Zealand, and replacing the local populations or whether CYP51 has

introgressed into a local genetic background. This hypothesis is tested and addressed with population genetic analysis using molecular markers in chapter 4.

Chapter 4: Population structure of *Zymoseptoria tritici* and dispersal of azole resistance across continents

4.1 Introduction

The sexual stage of *Zymoseptoria tritici* has been reported to start the initial infection in most countries included in this study (Madariaga, 1986; Shaw, 1987; Schuh, 1990; Cordo *et al.*, 1999). Ascospores are dispersed by the wind at long distances and infect the first wheat leaves close to the soil. Early sowing of wheat with a warm wet winter and a temperate humid spring are the ideal conditions for STB development. Subsequently, asexual pycnidiospores, locally dispersed by splash are predominant during most of the crop cycle (Eyal *et al.*, 1987; Eriksen *et al.*, 2001). The presence of sexual reproduction and a consistently large effective population size because of the huge areas sown to wheat, in addition to an extremely plastid genome, are responsible for the high variability observed in *Z. tritici* populations (Chen & McDonald, 1996). The distribution of genetic variation within and among populations is known as population structure. Genetic differentiation may be observed among populations of *Z. tritici*, depending on founder effects, migration patterns and variation in recombination frequency. In areas where sexual reproduction is rare, linkage disequilibrium may build up. High levels of migration and gene flow, by contrast, tend to move the species into a single panmictic population (Milgroom, 2017a). By contrast, in geographically isolated populations, restricted migration leads to genetic differentiation because of mutations, random genetic drift, and selection of specific alleles. For example, fungicide resistance alleles that arise by mutation in one isolated population are likely to be found in others in a different genetic background. As the degree of genetic differentiation depends on mutation, differential selection, genetic drift, and migration among populations, the study of it across the genome can be used to infer the processes that led to the evolution of fungicide resistance in different populations. To separate selection from other effects it is best to estimate the genetic structure with neutral markers that can be assumed not to be under selection of themselves. As an example of the insights this approach can bring, pathogen populations with high genetic variation

have higher evolutionary potential to adapt to adverse conditions such as fungicide applications in comparison to a population with low genetic variation (McDonald & Linde, 2002). The mode of reproduction has direct consequences for genetic variation and fungal evolution. Therefore estimating predominant reproduction modes is essential to understand the adaptation of plant-pathogen populations to environments of fungicide selection pressure (Milgroom, 2017b).

Using DNA restriction fragment length polymorphism (RFLP) as markers for genetics analysis of field isolates, earlier studies indicated that regional populations of *Z. tritici* were genetically very similar (Chen & McDonald, 1996; Linde *et al.*, 2002). Microsatellite or single sequence repeat (SSR) markers have since been used to study the genetics of *Z. tritici* populations from global to small spatial scale (Banke & McDonald, 2005; El Chartouni *et al.*, 2012; Siah *et al.*, 2018; Morais *et al.*, 2019). These markers are very highly polymorphic and allele-specific, which makes them ideal for population genetic analysis (Milgroom, 2017b). Genetic studies of worldwide populations of *Z. tritici* (Linde *et al.*, 2002; Zhan *et al.*, 2003; Jürgens *et al.*, 2006) have found high levels of genetic variability within regions, which along with large population sizes provides optimum conditions for the emergence of fungicide resistant alleles. Moreover, the presence of sexual reproduction ensures the generation of novel allele combinations through genetic recombination (Zhan & McDonald, 2004). In South America, molecular analysis revealed high genetic diversity in Argentinian populations of *Z. tritici*, with a panmictic overall population generated by high gene flow between regions (Castillo *et al.*, 2010).

In the previous chapters, the presence of azole resistance in South American populations of *Z. tritici* was reported. Moreover, the CYP51 amino acid changes present in the least sensitive strains were encoded by *CYP51* genes with a European genetic background. Therefore, in the present chapter, the hypothesis to be tested is that European strains of *Z. tritici* have migrated into South America and then recombined within local populations, leading to introgression of the azole-resistant alleles in an otherwise largely local genomic background. The alternative hypothesis is that European isolates are

actually replacing the local population. To test these hypotheses, the genetic diversity in and between populations from Europe, North and South America was analysed using microsatellite markers.

4.2 Material and methods

4.2.1 Fungal collection

One hundred and ninety-two isolates of *Z. tritici* were randomly selected from the populations Rothamsted-UK, Chile, Argentina, and Oregon-USA to assess the genetic diversity and structure of the pathogen population on a worldwide scale. Although sampled in the same continent, sites in Chile and Argentina were located over 1300 km apart. Forty-eight isolates from each of these populations were randomly selected within the whole collection discussed in previous chapters. In each location, leaves were sampled at untreated and naturally infected wheat plants in 2016. Isolation procedures and DNA extraction were as previously described in chapters 2 and 3.

4.2.2 Microsatellite genotyping

Isolates were genotyped with 16 microsatellites developed by Gautier *et al.* (2014) and described in Table 4.1. Preliminary experiments were carried out to adjust PCR conditions and multiplex markers in a way that each multiplex only contained non-overlapping allele sizes. These multiplexes were then tested with a selection of the worldwide isolates described in the methods chapter to check for the amplification and polymorphism of allele sizes (data not shown). The chosen markers were selected because they cover all 13 chromosomes on the core genome of *Z. tritici* and were found to be polymorphic in a worldwide population of this pathogen (Gautier *et al.*, 2014). Three extra microsatellites, described but not tested in Gautier *et al.*, (2014), were chosen for their strategic position around the *CYP51* gene at chromosome 7. In total, four multiplexes were needed to accommodate the 14 markers such that they could be amplified with the same PCR conditions. Microsatellites ST3A and ST7.4 were amplified in single reactions due to PCR incompatibilities. Amplifications were performed in a 10 µL reaction volume containing 24 ng of template DNA, 0.1 µM of each fluorescently labelled forward and common reverse primers, 10 mM Tris-HCl, 50 mM KCl, 1.5

mM $MgCl_2$, 0.2 mM of each dNTP, and 0.25 U of Taq DNA polymerase (Sigma-Aldrich, United Kingdom) in a thermal cycler (SureCycler 8800; Agilent Technologies, USA). PCR conditions were used like the following program: 94°C for 2 min, then 40 cycles of 94°C for 30 s, 57°C for 90 s, 72°C for 1 min, and a final extension step of 72°C for 5 min. The annealing temperature for marker ST7.4 was 59°C. Forward primers were labelled with either PET, NED, 6-FAM or VIC fluorescent dyes (Applied Biosystems, United Kingdom) (Table 4.1).

PCR products were visualized on 2.0% agarose gel to check for amplification products. Amplicons of markers ST3A and ST7.4 were pooled together with those from multiplexes 3 and 4, respectively, for analysis. Mixtures of 1 µL of PCR products with 8.8 µL of Hi-Di™ formamide (Thermo Fisher Scientific, United Kingdom) and 0.2 µL of GeneScan™ 500 LIZ™ (Applied Biosystems, United Kingdom) were denatured at 94°C for 5 min. Amplified microsatellite fragments were electrophoretically separated in a 3130xl genetic analyser (Applied Biosystems, United Kingdom). Raw data were collected and converted into allele sizes using GeneMapper™ software v4.0 (Applied Biosystems, United Kingdom).

Table 4.1 Primer characteristics and multiplex compositions of 16 microsatellites for *Zymoseptoria tritici* population genetic studies.

Multiplex	Locus	Chromosome	Repeat Motif	Primer sequences (5'-3')	Fluorescent dye	Annealing temperature
1	ST6	6	(TCC)8	F: TCAATTGCCAATAATTCGGG R: AGACGAGGCAGTTGGTTGAG	Red-PET	57°C
	ST1	1	(GAC)6	F: AATCGACCCCTTCCTTCAAC R: GGGGGAGAGGCATAGTCTTG	Yellow-NED	57°C
	ST5	5	(GGC)8	F: GATACCAAGGTGGCCAAGG R: CACGTTGGGAGTGTCGAAG	Green-VIC	57°C
	ST9	9	(CT)6	F: CACCTCACTCCTCAATTCCG R: GAAAGGTTGGTGTCGTGTCC	Blue-FAM	57°C
2	ST13	13	(AG)10(GGCA)3	F: GACTCCATTTACCTGTGGCG R: TGTGAAGGACACGCAAAGAG	Red-PET	57°C
	ST12	12	(TCC)6	F: GAATCCACCTCTTCCTTGCC R: AGGAGGATATCAAGGCCCAG	Yellow-NED	57°C
	ST8.2	8	(AGG)5 (GAG)16 (AGG)5 (AGG)5	F: CGTCCTCAGTTACTCCGCTC R: CTCCGTCACTCGCTAAGACC	Green-VIC	57°C
	ST7.2	7	(CGT)5	F: GTGGAGCACTTGCTTTGTGA R: CGCGGCGAGATCTACTACTC	Blue-FAM	57°C

Table 4.1 cont.

Multiplex	Locus	Chromosome	Repeat Motif	Primer sequences (5'-3')	Fluorescent dye	Annealing temperature
	ST4	4	(AC)7	F: TGAACATCAACCTCACACGC R: AGAAGAGGACGACCCACGAG	Red-PET	57°C
3	ST7.5	7	(TGCC)3 (ACGA)3	F: TGCTCAGGCGATTACAACAC R: ATGCAAGCAGCAATGACAAG	Green-VIC	57°C
	ST2	2	(GCT)12	F: ACACCAAAGAAGGATCCACG R: GCCGGAGGTCTATCAGTTTG	Blue-FAM	57°C
	ST10	10	(CAA)7	F: TCCGTCATCAACAACACCAG R: TGGCCGTAGAACTGCTGAG	Red-PET	57°C
4	ST7	7	(AC)21	F: CACCACACCGTCGTTCAAG R: CGTAAGTTGGTGGAGATGGG	Yellow-NED	57°C
	ST11.1	11	(ATC)11	F: ACAATAGCCAGGGAATCGTG R: ATTTGGAGTGGTGGTGGTTC	Green-VIC	57°C
	ST3A	3	(AG)15	F: ACTTGGGGAGGTGTTGTGAG R: ACGAATTGTTCATTCCAGCG	Yellow-NED	57°C
	ST7.4	7	(GAG)5	F: CACCAGCAACACAGCAACTT R: GTGACGGGGATCTTCTTTGA	Blue-FAM	59°C

4.2.3 Data analysis

A multi-locus Genotype (MLG) code was constructed for each *Z. tritici* isolate according to the observed allele sizes across 12 microsatellite loci. As expected for a haploid organism, only one allele was amplified per locus. Individuals with the same alleles across all loci were treated as clones of the same MLG. Genetic diversity analysis was performed using the complete data set of *Z. tritici* isolates. GenAlex v.6.5 software (Peakall & Smouse, 2012) was used to generate a clone-corrected data set by detecting isolates with identical MLGs within each population and maintaining only one representative individual per MLG. To minimize the effects of clonal reproduction during epidemics the clone-corrected data set was used for all other populational genetic analyses.

4.2.3.1 Allelic and genotypic diversity

GenAlex v.6.5 software was used to calculate allelic diversity: the number of different alleles (N_A) averaged across loci; Nei's gene diversity (*H*) (Nei, 1973) and Nei's unbiased gene diversity (*uH*). Allelic richness (A_R) estimated the expected number of alleles in a subsample of 37 genotypes corresponding to the sample size of the smallest population, based on 1,000 permutations, as implemented in FSTAT v.2.9.4. software (Goudet, 1995).

Genotypic diversity was calculated as the fraction of different genotypes in the sample, and clonal fraction estimated per population as [1 - Genotypic diversity]. Stoddart and Taylor's genotypic diversity index (*G*) (Stoddart & Taylor, 1988) was estimated for each population using the *poppr* package v.2.8.3 in R environment (Kamvar *et al.*, 2015). Stoddart and Taylor's genotypic index is proportional to sample sizes, therefore the same package was also used to calculate Simpson's index of genotypic diversity (λ) and genotypic Evenness (*E*) (Simpson, 1949; Grünwald *et al.*, 2003).

4.2.3.2 Population differentiation

The distribution of genetic diversity using the clone-corrected data set within and among *Z. tritici* populations was estimated by a hierarchical analysis of molecular variance (AMOVA), as implemented in Arlequin v.3.5 software (Excoffier & Lischer, 2010), with significance testing by using 1,000

permutations. To compare differentiation among pairs of populations both F_{st} and PhiPT (ϕ_{PT}) indices (Meirmans & Hedrick, 2011) were calculated using the software Arlequin and GenAlex, respectively.

The population structure was analysed using Structure v.2.3.4 software (Pritchard *et al.*, 2000). This model-based Bayesian approach estimates the existence of divergent genetic clusters and assigns probabilistically individuals to populations. The software was run using an admixture ancestry model-based clustering method, allowing mixed ancestry among individuals from different populations (*K*) with correlated allele frequencies among populations. Ten independent runs were performed using 5,000,000 iterations of the Markov Monte Carlo Chain (MCMC) used as burn-in followed by 500,000 MCMC iterations, assuming 1 to 7 populations. The best estimate of *K* populations was assessed based on the maximum log probability of data Ln*P*(*D*) for different values of *K* by using the statistic ΔK (Evanno *et al.*, 2005). The website Structure Harvester (Earl & VonHoldt, 2012) was used for visualizing Structure output and implementing the Evanno method (Evanno *et al.*, 2005).

The discriminant analysis of principal components (DAPC), a non-model-based clustering method, was used to confirm the pattern of population subdivision inferred using Structure. This method maximizes variation between groups, minimizing the within-group genetic variability; additionally, it does not require assumptions regarding evolutionary models (Jombart *et al.*, 2010). DAPC was carried out in the R environment using the Adegenet package v.2.1.1 (Jombart & Ahmed, 2011). The first 100 principal components were retained. Ten separate runs of *K*-means were performed. The mean Bayesian information criterion (BIC) value at each value of *K* from 1 to 15 was plotted. Furthermore, a distance-based tree was generated using the UPGMA method with the software Mega X v.10.0.5 (Kumar *et al.*, 2018) to infer relationships among a selection of 42 isolates with known CYP51 variants. Additionally, associations among MLGs carrying these CYP51 variants were assessed with a minimum spanning network (MSN). The matrix of Nei's genetic distance and MSN were generated with *poppr* package v.2.8.3 in R environment (Kamvar *et al.*, 2015).

The directional relative migration network among populations was determined using the divMigrate online software (Sundqvist *et al.*, 2016). Jost's *D* (D_{est}) was used as a measure of genetic differentiation with a bootstrap value of 1,000 (Jost, 2008). The network plots were used to visualize patterns of directional relative migration and gene flow among populations as in Siah *et al.* (2018). Finally, the potential isolation-by-distance between populations was tested by the relationship between geographic and genetic distance with the nonparametric Mantel test. The matrix of pairwise genetic differentiation between locations (with ϕ_{PT} index) and a matrix of geographic distance, obtained from Lat/Long coordinates in decimal degrees, were tested using GenAlex v.6.5 software with 1,000 random permutations (Peakall & Smouse, 2012; Siah *et al.*, 2018).

4.2.3.3 Reproductive mode

The index of association (I_A) was used to assess the predominant reproduction strategy of the pathogen. This index estimates the multilocus linkage disequilibrium; however, the magnitude of I_A depends on the number of loci studied. Therefore the r_D, a locus number independent measure, was also measured (Agapow & Burt, 2001). Values of r_D close to zero indicate random mating, while higher values indicate gametic disequilibrium as expected in asexual or inbreeding populations. These indices were calculated with the clone-corrected data set in the R package *poppr*, while 1,000 permutations were used to detect if the observed values deviated significantly from the null hypothesis of no linkage disequilibrium among loci.

4.3 Results

4.3.1 Summary of microsatellite variability and allelic/genotypic diversity

Four samples, each containing 48 isolates of *Z. tritici* were collected from populations in individual fields from the United Kingdom (Rothamsted), Chile, Argentina, and the United States (Oregon). All 192 isolates were characterized using microsatellite markers to assess genetic diversity and population structure of the pathogen. Three markers located on chromosome 7 (ST7.2, ST7.4, and ST7.5) did not show polymorphisms, while one marker in chromosome 11 (ST11.1) showed 45% of null alleles.

Therefore, these four markers were excluded from this study, leaving 12 microsatellite markers to be analysed.

In total, 83 alleles were scored across 12 markers within the entire collection of isolates. The number of alleles per loci ranged from 2 (ST1) to 13 (ST7) (Table 4.2), with a mean of 3.92 ± 0.28 (standard error) alleles per locus over populations and loci (Table 4.4). The effective number of alleles ranged from 1.16 ± 0.05 for locus ST12 to 3.79 ± 0.71 in locus ST3A. The locus ST12 had the lowest unbiased Nei's diversity (0.13 ± 0.05), while ST3A was the locus with the highest value for this index (0.72 ± 0.06) (Table 4.3). The power of the 12 loci to detect all MLGs was tested by calculating a genotype accumulation curve in poppr v.2.8.3 (Kamvar *et al.*, 2014). The genotype accumulation curve tended to plateau as the number of sampled loci increased, confirming that all 12 markers were needed, but sufficient, for MLG discrimination (Figure 4.1).

Considering the populations, the number of alleles ranged from 3.50 in Chile and Argentina to 5.08 in Rothamsted. The allelic richness and gene diversity were also measured across the markers to each population. Oregon showed the highest number of alleles based on the smallest population size (n = 37, because individuals with a null allele at any loci were not considered), while Argentina had the highest value of gene diversity (Table 4.4). Unbiased Nei's gene diversity index values, corrected for sample size, were slightly higher than the Nei's index. Nevertheless, both indices displayed a similar extent of genetic diversity among populations.

A total of 182 MLGs were found within 192 isolates, which represents an overall clonal fraction of less than 5%. Genotypic diversity was high in all populations (Table 4.4). Although there were no MLG clones in the Argentinian sample, it shared one MLG with Chile. The highest estimate of Stoddart's genotypic diversity was observed in Argentina. While Simpson's index of genotypic diversity was similar among populations and across all collections of isolates, evenness was slightly higher in Argentina (Table 4.4).

Table 4.2 Sample size and number of alleles by locus markers in *Zymoseptoria tritici* field populations from Chile, Argentina, Oregon-USA and Rothamsted-UK.

Population		ST1	ST2	ST3A	ST4	ST5	ST6	ST7	ST8.2	ST9	ST10	ST12	ST13
Chile	n[1]	48	48	48	48	48	48	48	48	48	48	46	48
	Na[2]	1	5	5	2	4	3	4	4	3	4	1	6
Argentina	n	48	48	48	48	48	48	48	44	48	48	48	48
	Na	2	4	4	3	2	3	6	5	2	5	2	4
Oregon	n	48	48	48	48	47	48	47	48	48	47	37	48
	Na	2	3	8	2	2	3	5	6	3	3	3	3
Rothamsted	n	48	47	47	48	47	47	47	46	48	45	48	47
	Na	2	6	9	4	4	3	10	7	4	5	3	4
Total	n	48	48	48	48	48	48	48	48	48	48	48	48
	Na	2	8	12	4	6	4	13	12	6	6	4	6

[1]Number of isolates.
[2]Number of different alleles.

Table 4.3 Genetic diversity estimated by SSR locus across the combined isolate set of *Zymoseptoria tritici* field populations from Chile, Argentina, Oregon-USA and Rothamsted-UK.

Locus	N_A[1]	N_E[2]	uH[3]
ST1	1.75 ± 0.25	1.37 ± 0.16	0.24 ± 0.09
ST2	4.50 ± 0.64	2.46 ± 0.49	0.55 ± 0.09
ST3A	6.50 ± 1.19	3.79 ± 0.71	0.72 ± 0.06
ST4	2.75 ± 0.48	1.88 ± 0.07	0.48 ± 0.02
ST5	3.00 ± 0.58	1.58 ± 0.07	0.37 ± 0.03
ST6	3.00 ± 0.00	1.82 ± 0.29	0.42 ± 0.09
ST7	6.25 ± 1.31	2.39 ± 0.29	0.57 ± 0.07
ST8.2	5.50 ± 0.64	1.53 ± 0.10	0.34 ± 0.05
ST9	3.00 ± 0.41	1.27 ± 0.08	0.21 ± 0.05
ST10	4.25 ± 0.48	2.19 ± 0.30	0.51 ± 0.09
ST12	2.25 ± 0.48	1.16 ± 0.05	0.13 ± 0.05
ST13	4.25 ± 0.63	2.78 ± 0.43	0.63 ± 0.05

[1]Mean number of distinct alleles ± standard error.
[2]Number of effective alleles ± standard error (Peakall & Smouse, 2012).
[3]Nei's unbiased gene diversity corrected for the sample size (Peakall & Smouse, 2012).

Table 4.4 Estimates of allelic and genotypic diversity based on 12 microsatellite markers in *Zymoseptoria tritici* field populations from Chile, Argentina, Oregon-USA and Rothamsted-UK.

Population	n[1]	Allelic				Genotypic					
		NA[2]	*H*[3]	*uH*[4]	A_R[5]	MLGs[6]	Genotypic diversity[7]	Clonal fraction[8]	*G*[9]	*λ*[10]	*E*[11]
Rothamsted-UK	48	5.08 ± 0.71	0.45 ± 0.06	0.46 ± 0.06	3.47	46	0.96	0.04	44.3	0.98	0.98
Chile	48	3.50 ± 0.45	0.37 ± 0.07	0.38 ± 0.07	3.48	45	0.94	0.06	38.4	0.97	0.90
Argentina	48	3.50 ± 0.40	0.48 ± 0.05	0.49 ± 0.05	3.54	48	1.00	0.00	48.0	0.98	1.00
Oregon-USA	48	3.58 ± 0.53	0.39 ± 0.06	0.40 ± 0.06	5.02	44	0.92	0.08	39.7	0.97	0.94
Total	192	3.92 ± 0.28	0.42 ± 0.03	0.43 ± 0.03	-	182	0.95	0.05	167.6	0.99	0.95

[1]Number of isolates.
[2]Number of different alleles averaged across loci ± standard error.
[3]Nei's gene diversity averaged over all loci ± standard error.
[4]Nei's unbiased gene diversity averaged all loci ± standard error.
[5]Allelic richness estimated by rarefaction to N = 37.
[6]Number of distinct multilocus genotypes.
[7]Number of distinct genotypes/sample size.
[8]1-Genotypic diversity.
[9]Stoddart and Taylor's Index of genotypic diversity.
[10]Simpson's index.
[11]Evenness.

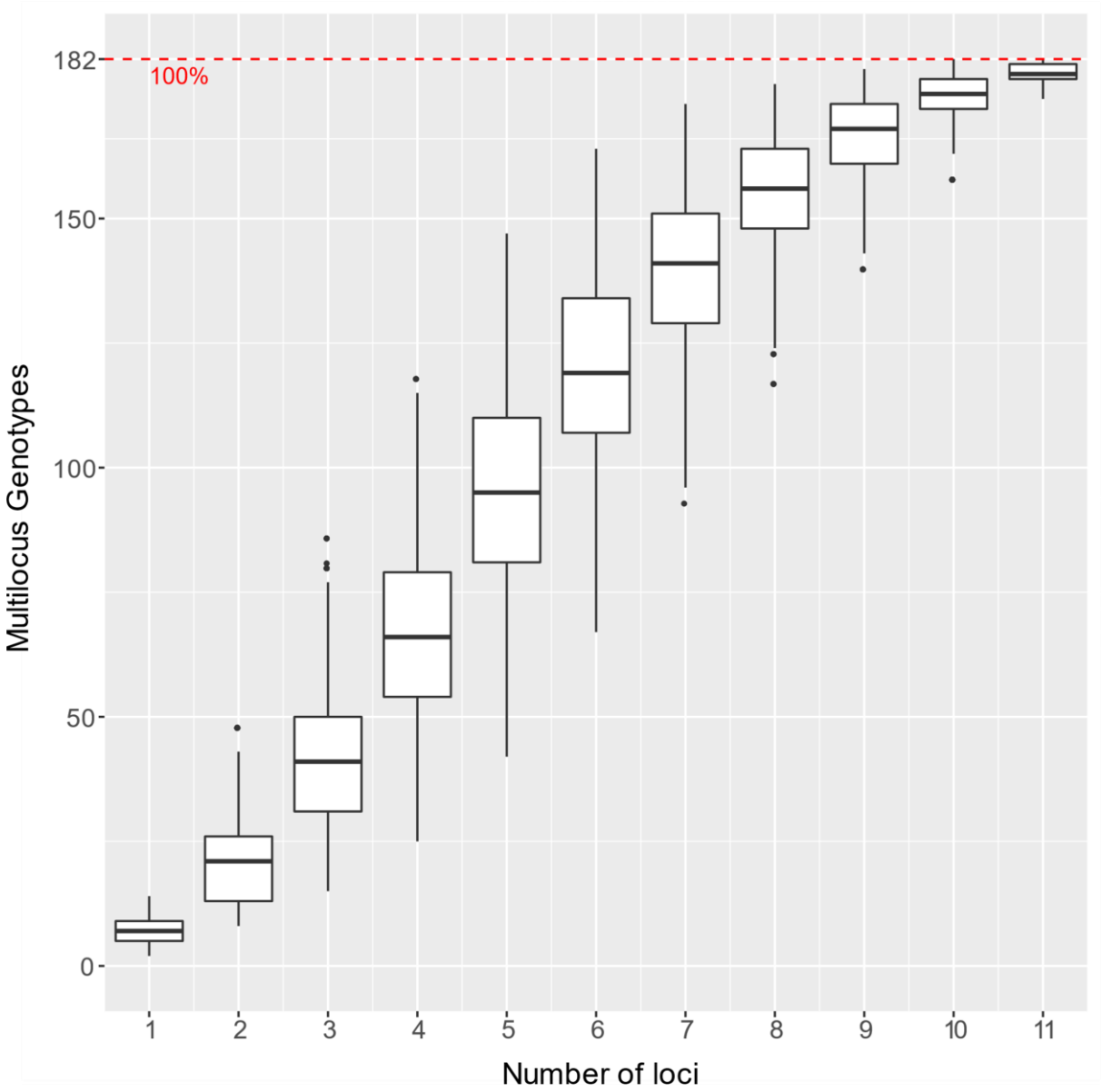

Figure 4.1 Genotype accumulation curve for 12 microsatellite loci for the combined isolate set of *Zymoseptoria tritici* field populations from Chile, Argentina, Oregon-USA and Rothamsted-UK.

4.3.2 Population differentiation

The hierarchical AMOVA was performed with the clone corrected data set so that only a single representative of each MLG was present in each population. Moderate genetic differentiation, accordingly to Wright (1978), was observed between populations (F_{ST} = 0.12; P < 0.001). 12.4% of the genetic variation could be explained by differences among populations, or equivalently 87.6% of global genetic diversity was present within populations. Pairwise comparisons among populations with

fixation indices F_{ST} and ϕ_{PT} showed significant differentiation between all populations (Null hypothesis of genetic differentiation, $P \leq 0.001$). The value ranges for both indices were similar and showed moderate genetic differentiation in all comparisons (Table 4.5).

Whilst AMOVA estimates F_{ST} for predefined subpopulations, clustering methods like Structure and DAPC, infer the subpopulations *de novo* from the clusters observed. The Bayesian clustering method implemented in Structure (Pritchard *et al.*, 2000) was used assuming a model with admixture and correlated allele frequencies to investigate the number of genetic clusters apparent in the data. The highest value of ΔK was observed with K = 2 (ΔK value = 89.7). Nevertheless, the value of ΔK for K = 4 also had high value (Figure 4.2). Therefore, the individual membership coefficients for each cluster were plotted for K = 2, K = 3, and K = 4 (Figure 4.3). Regardless of the value of K, all individuals displayed different levels of admixed membership to the proposed genetic clusters. Observing the overall distribution of clusters from K = 2 to K = 4 it was possible to identify how they were differentially dispersed among populations. With k = 2 the South American populations displayed an overall similar distribution of membership, rather different from Oregon and Rothamsted. Nevertheless, some genotypes from Rothamsted were more affiliated with those from South America (Figure 4.3 A). With the introduction of a third cluster, K = 3, Oregon became more distinct from Rothamsted (Figure 4.3 B). Lastly, with four clusters, differentiation between populations was clear, for each sampling location was assigned one predominant colour (Figure 4.3 C). Note that the clustering process itself ignores geographic structure in the data. This analysis revealed a biologically relevant structure and supported the existence of genetic differentiation between populations. There was only slight differentiation between the two South American regional populations, while Oregon was distinct from the other three sample locations.

Additionally, genetic similarity among individuals using DAPC was estimated to infer clusters without considering previous population criteria. The DAPC was calculated with the first 100 principal components that captured 100% of the variation and then the first three discriminant functions

retained. K-means clustering was applied to infer K as the number of clusters, with the optimal number of clusters determined by the Bayesian information criterion (BIC) (Figure 4.4). The lowest value of BIC was observed with K = 7 clusters, while the elbow in the curve was observed at K = 3. However, four clusters (K = 4) were used to allow population structure to emerge since there were four regions in the data. Although the resulting DAPC with four clusters suggested distinct MLG clustering accordingly to sampling locations, considerable overlap between all four populations was also observed (Figure 4.5). All four clusters included MLGs from all four populations, though in different proportions. Most South American isolates were in clusters 1 and 3, separated from other clusters on the first discriminant axis, which represented 87.4% of the discriminatory power. While cluster 1 had 47% of the Chilean isolates, cluster 3 had 48% of the Argentinian isolates. Cluster 2 had 64% of the Oregon isolates and was separated from the other clusters on the second discriminant axis, which represented 8.6% of the discrimination power. Cluster 4 represented 74% of Rothamsted isolates (Figure 4.5).

The first cluster (Cluster 1) evidenced by DAPC had isolates from the four populations, but mostly (50%) of these isolates were from Chile. However, clustering of CYP51 haplotypes or related CYP51 variants was not observed (Figure 4.5; Table 4.6). For example, the V136A, S188N, Δ459 & Δ460 variant was found in four isolates from Chile, all in different clusters, while four L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K haplotypes from Chile were observed in three different clusters (Table 4.6). This was in accordance with the dendrogram constructed with those isolates with known CYP51 sequence, in which isolates from Chile did not group by CYP51 haplotypes (Figure 4.6). Similar behaviour was observed in Rothamsted. Cluster 4 was composed of 51% European strains, but those isolates from Rothamsted with identical CYP51 sequences grouped in separated clusters of the DAPC and dendrogram.

Oregon and Argentina compose the majority of clusters 2 (72%) and 3 (66%) of the DAPC, respectively. Nonetheless, they also had isolates in all four clusters (Figure 4.5). As in Chile and Rothamsted, CYP51 haplotypes from Oregon and Argentina grouped in separated clusters of DAPC and dendrogram.

Isolates from Oregon and Argentina tended to be more individually grouped than those from Rothamsted and Chile in the dendrogram (Figure 4.6). Interestingly, isolate ORE.41, with CYP51 variant I381V & Y461S, was separated from all other isolates in both DAPC and dendrogram, even from the other three isolates from Oregon with this same amino acid variant and synonymous haplotype.

The minimum spanning network showed that 40 MLGs from the different populations formed a single large group with no MLG separated by long distances (Figure 4.7). Reticulations in the network were present among MLGs from all populations, indicating multiple paths between MLGs. There were only two clonal MLGs, both found in Oregon, with two individuals each. No cluster was exclusively formed of isolates from within a single region. Rothamsted was the population with the maximum number of connected MLGs, with 10 out of 12 MLGs directly connected. Some clusters strongly associated with specific regions were observed in all regions, but MLGs from all four populations were spread throughout the network. Moreover, MLGs with the same CYP51 haplotype did not cluster together. This pattern was also observed in both the DAPC and the dendrogram, where clusters were not formed exclusively by any single population or CYP51 haplotype.

A directional relative migration network revealed gene flow only from Chile to Argentina (D = 1; p = 0.05) (Figure 4.8). Although a Mantel test revealed a positive correlation (Rxy = 0.43) between genetic distances (ϕ_{PT}) and the logarithm of geographic distances, the isolation-by-distance (IBD) hypothesis was not strongly supported (Null hypothesis of IBD, P = 0.16).

Table 4.5 Population differentiation measured by F_{ST} (below the diagonal) and PhiPT (ϕ_{PT}) (above the diagonal) among the four *Zymoseptoria tritici* field populations from Chile, Argentina, Oregon-USA and Rothamsted-UK.

Population	Rothamsted-UK	Chile	Argentina	Oregon-USA
Rothamsted-UK	…	0.095*	0.099*	0.111*
Chile	0.101*	…	0.090*	0.168*
Argentina	0.107*	0.092*	…	0.151*
Oregon-USA	0.117*	0.172*	0.157*	…

*Significant genetic differentiation (P ≤ 0.001) in pairwise comparisons based on 1,000 permutations.

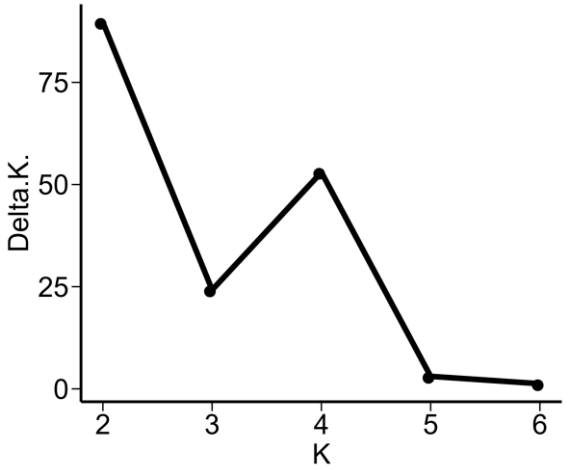

Figure 4.2 The most likely number of clusters (*K*) based on Evanno's ΔK method.

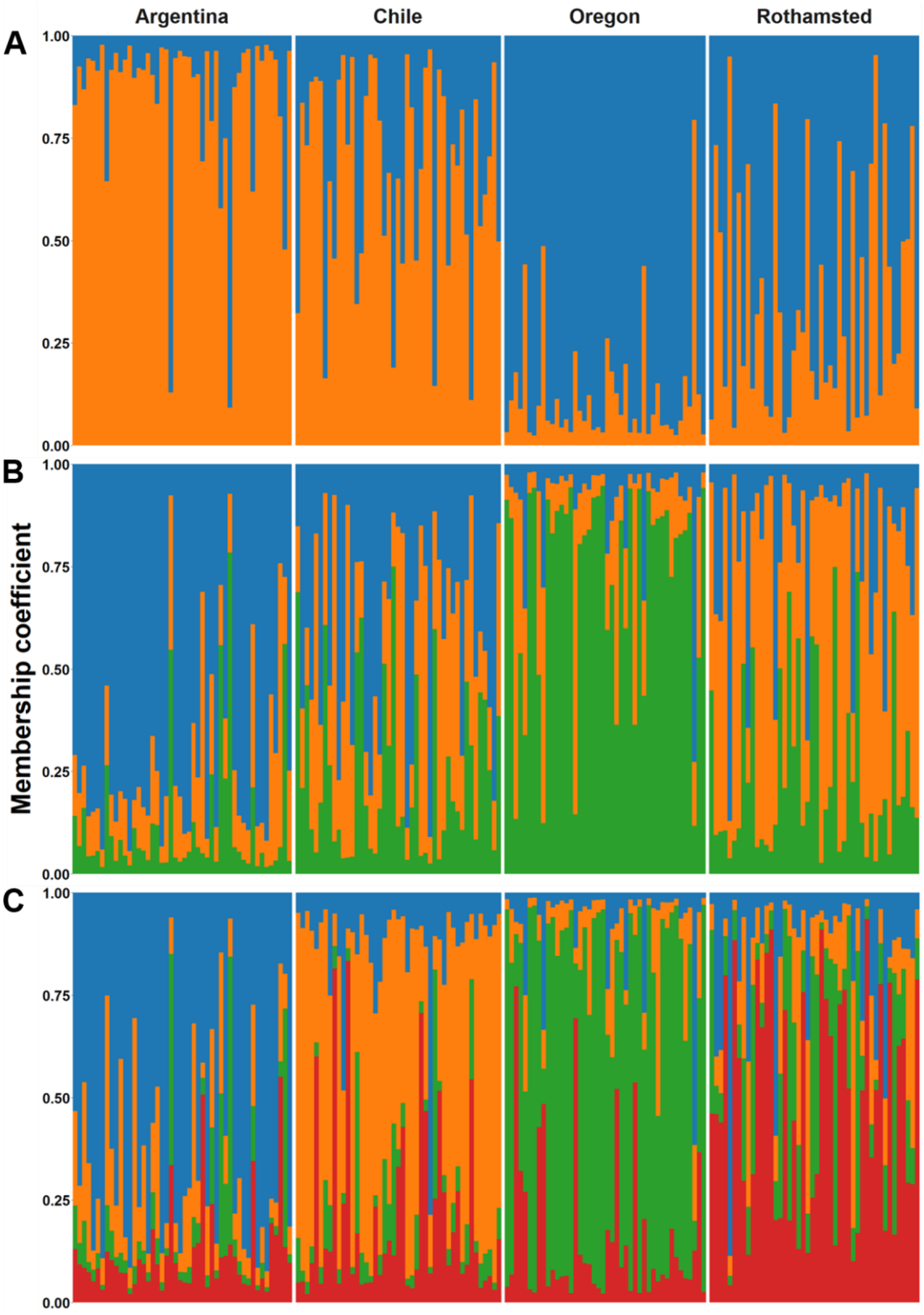

Figure 4.3 Bar plot from Bayesian inference cluster analysis showing the distribution of 183 MLG assignations estimated for K = 2 clusters (A), K = 3 clusters (B) and K = 4 clusters (C). Individuals are grouped according to their sampling locations. Each vertical line represents an individual and the length of each coloured line corresponds to the membership coefficient for each cluster (Orange, Blue, Green, and Red).

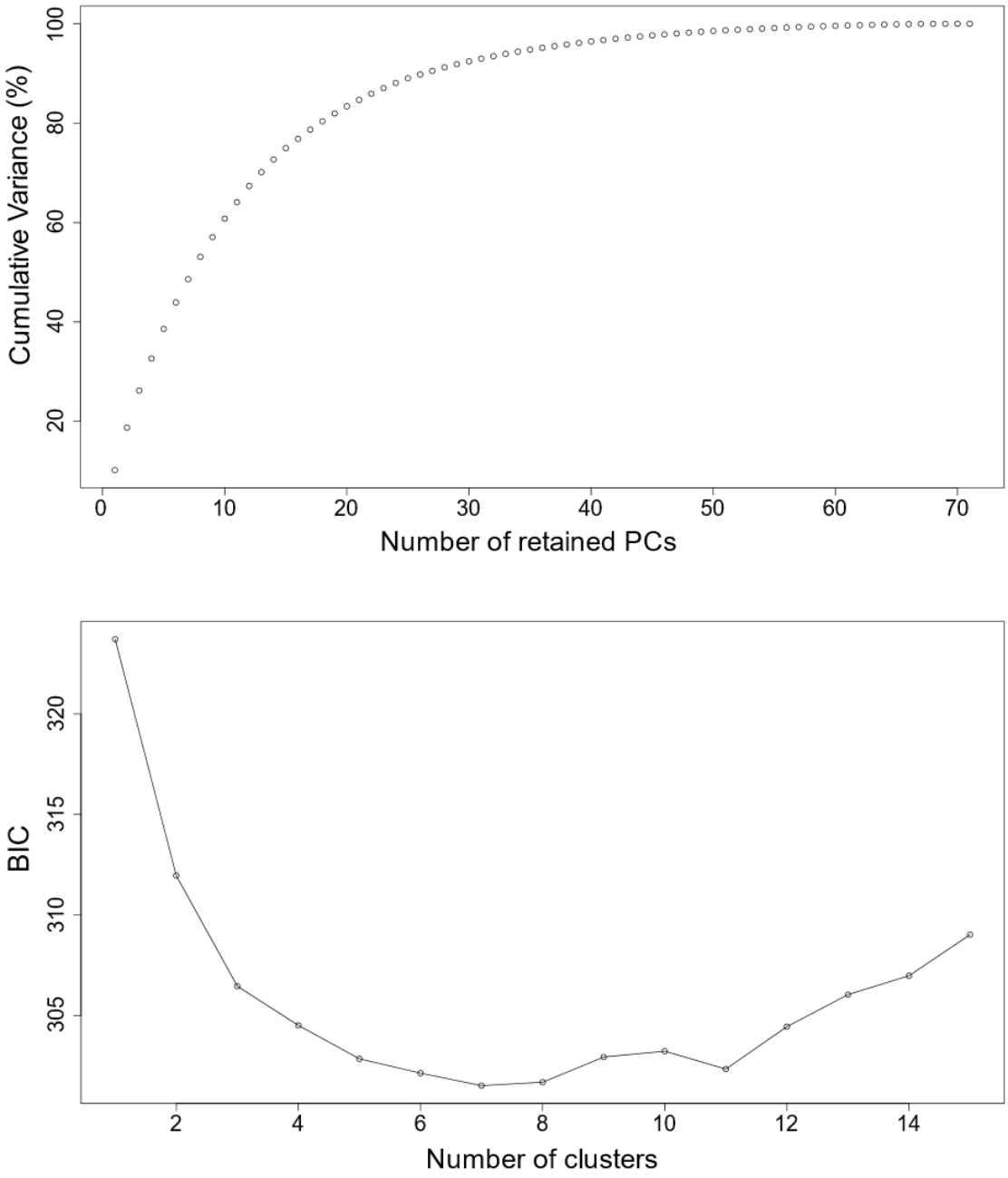

Figure 4.4 Number of retained Principal Components and Bayesian Information Criterion.

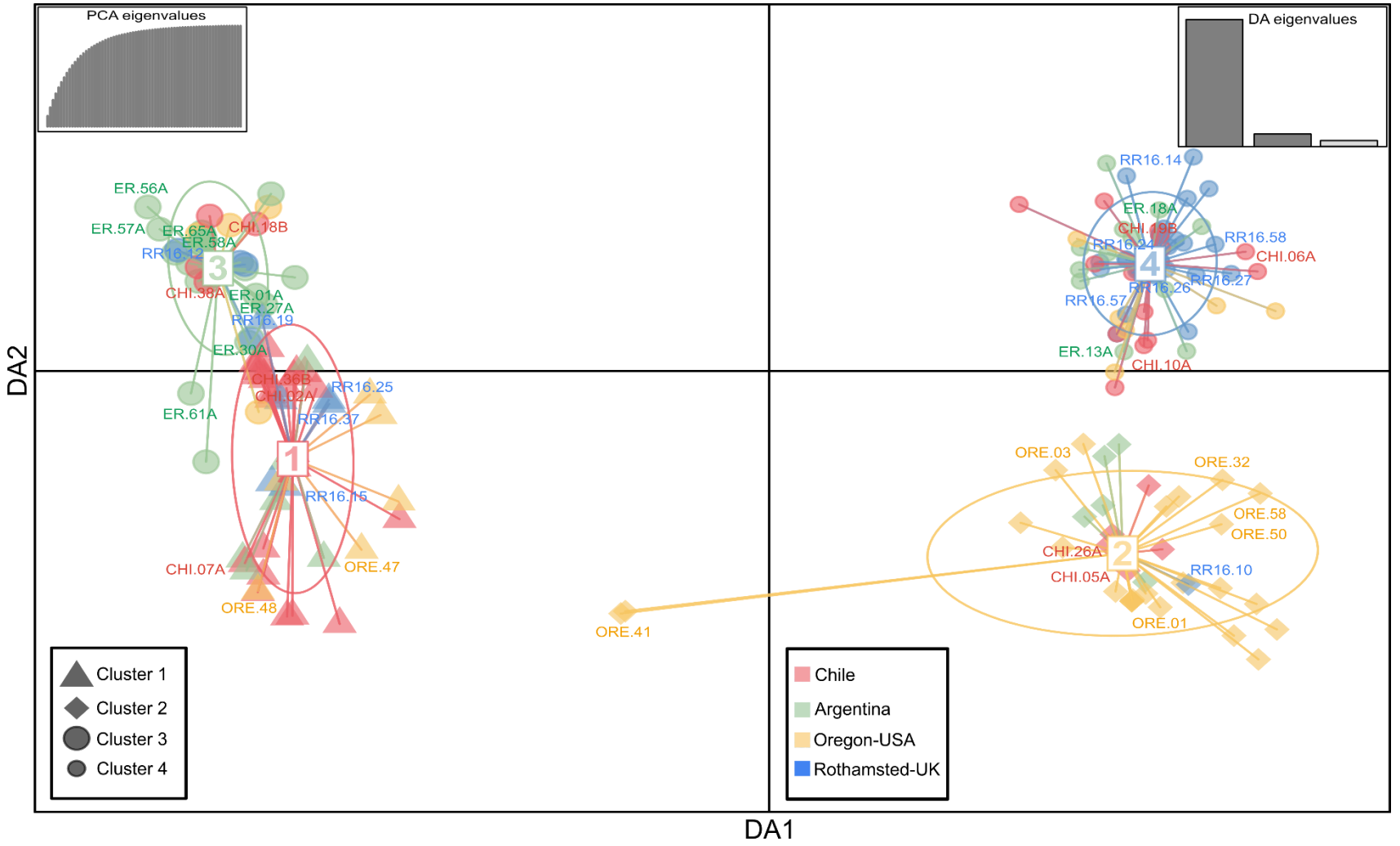

Figure 4.5 Discriminant analysis of principal components (DAPC) for 183 MLGs of *Zymoseptoria tritici* sampled in Chile (CHI-Red), Argentina (ER-Green), Oregon-USA (ORE-Orange) and Rothamsted-UK (RR16-Blue). Each cluster represents different subpopulations identified by DAPC analysis. Bar plots represent the eigenvalues of the first three discriminant axes.

Table 4.6 CYP51 variants associated with *Zymoseptoria tritici* field isolates included in DAPC analysis.

Population	Isolate	CYP51 variant	DAPC Cluster
Rothamsted-UK	R16.58	V136C, I381V, Y461H & S524T	4
	R16.14	L50S, D134G, V136A, I381V & Y461H	4
	R16.15	L50S, D134G, V136A, I381V & Y461H	1
	R16.27	L50S, D134G, V136A, I381V & Y461H	4
	R16.12	L50S, V136A, I381V, Y461H & S524T	3
	R16.24	L50S, V136C, S188N, I381V, Y461H & S524T	4
	R16.25	L50S, S188N, I381V, Δ459, Δ460 & N513K	1
	R16.26	L50S, S188N, I381V, Δ459, Δ460 & N513K	4
	R16.37	L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K	1
	R16.10	L50S, V136A, S188N, A379G, I381V, Δ459, Δ460 & S524T	2
	R16.19	L50S, V136A, S188N, A379G, I381V, Δ459, Δ460 & S524T	3
	R16.57	L50S, D134G, V136A, A379G, I381V, Δ459, Δ460, N513K & S524T	4
Chile	CHI.2A	S188N & N513K	1
	CHI.5A	V136A, S188N, Δ459 & Δ460	2
	CHI.7A	V136A, S188N, Δ459 & Δ460	1
	CHI.10A	V136A, S188N, Δ459 & Δ460	4
	CHI.38A	V136A, S188N, Δ459 & Δ460	3
	CHI.26A	L50S, S188N, Δ459, Δ460 & N513K	2
	CHI.6A	L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K	4
	CHI.18B	L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K	3
	CHI.19B	L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K	4
	CHI.36B	L50S, S188N, A379G, I381V, Δ459, Δ460 & N513K	1

Table 4.6 cont.

Population	Isolate	CYP51 variant	DAPC Cluster
Argentina	ER.58A	Wild type	3
	ER.65A	Y459H	3
	ER.01A	Y461S	3
	ER.27A	Y461H	3
	ER.13A	L50S, I381V & Y461H	4
	ER.18A	L50S, I381V & Y461H	4
	ER.56A	L50S, I381V & Y461H	3
	ER.57A	L50S, I381V & Y461H	3
	ER.30A	L50S, S188N, A379G, I381V, Δ459/460 & N513K	3
	ER.61A	L50S, S188N, A379G, I381V, Δ459/460 & N513K	3
Oregon-USA	ORE.01	G460D	2
	ORE.50	G460D	2
	ORE.47	L50S & Y459D[1]	1
	ORE.44	L50S & Y459D[1]	1
	ORE.32	V136A & Y461S	2
	ORE.48	V136A & Y461S	1
	ORE.03	I381V & Y461S	2
	ORE.07	I381V & Y461S[2]	2
	ORE.41	I381V & Y461S	2
	ORE.58	I381V & Y461S[2]	2

[1]ORE.44 and ORE.47 were clonal MLGs.
[2]ORE.07 and ORE.58 were clonal MLGs.

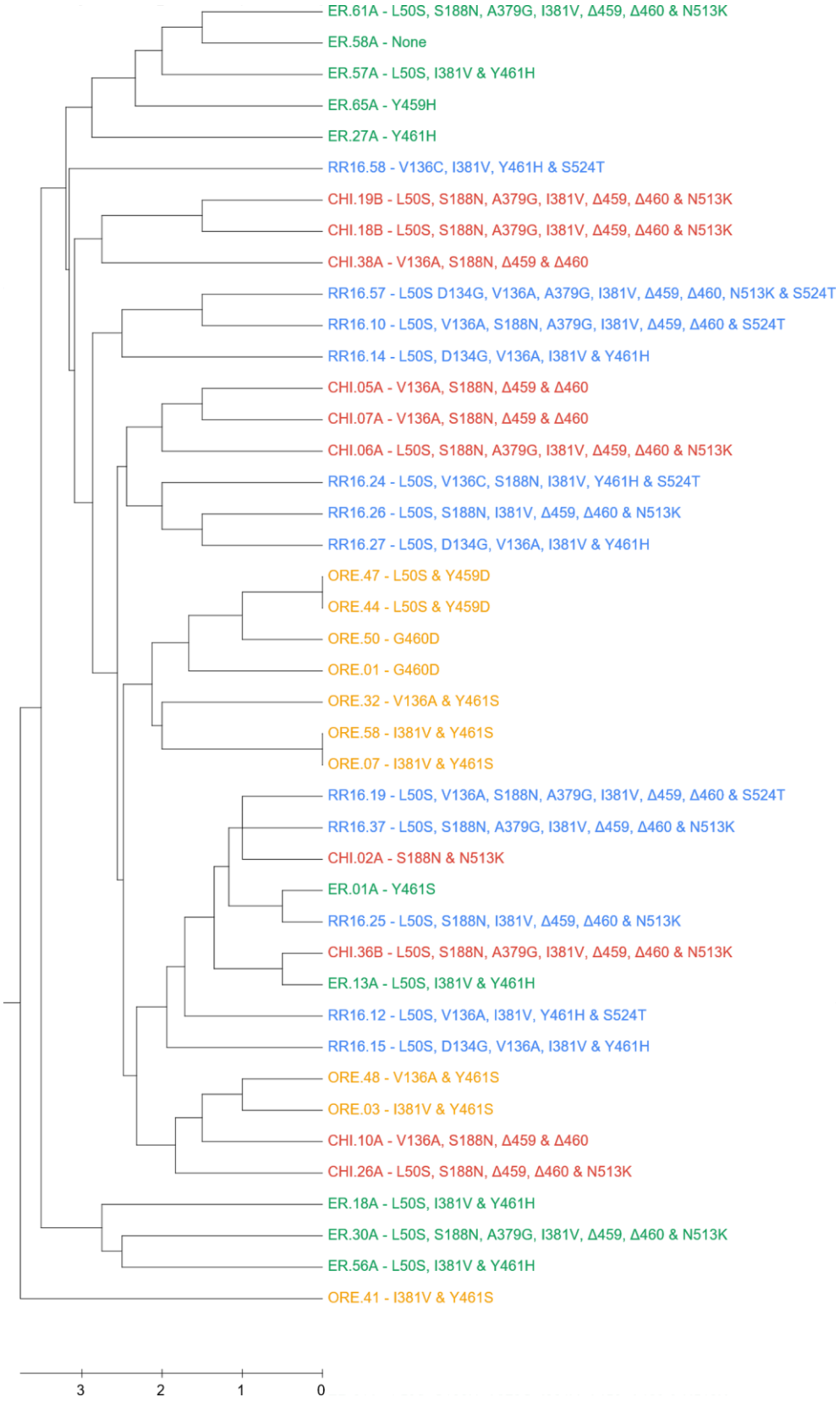

Figure 4.6 UPGMA dendrogram of haploid genetic distance (Peakall & Smouse, 2012) for 42 field isolates of *Zymoseptoria tritici* from Chile (CHI-Red), Argentina (ER-Green), Oregon-USA (ORE-Orange) and Rothamsted-UK (RR16-Blue). CYP51 variants associated with each isolate are described alongside its code.

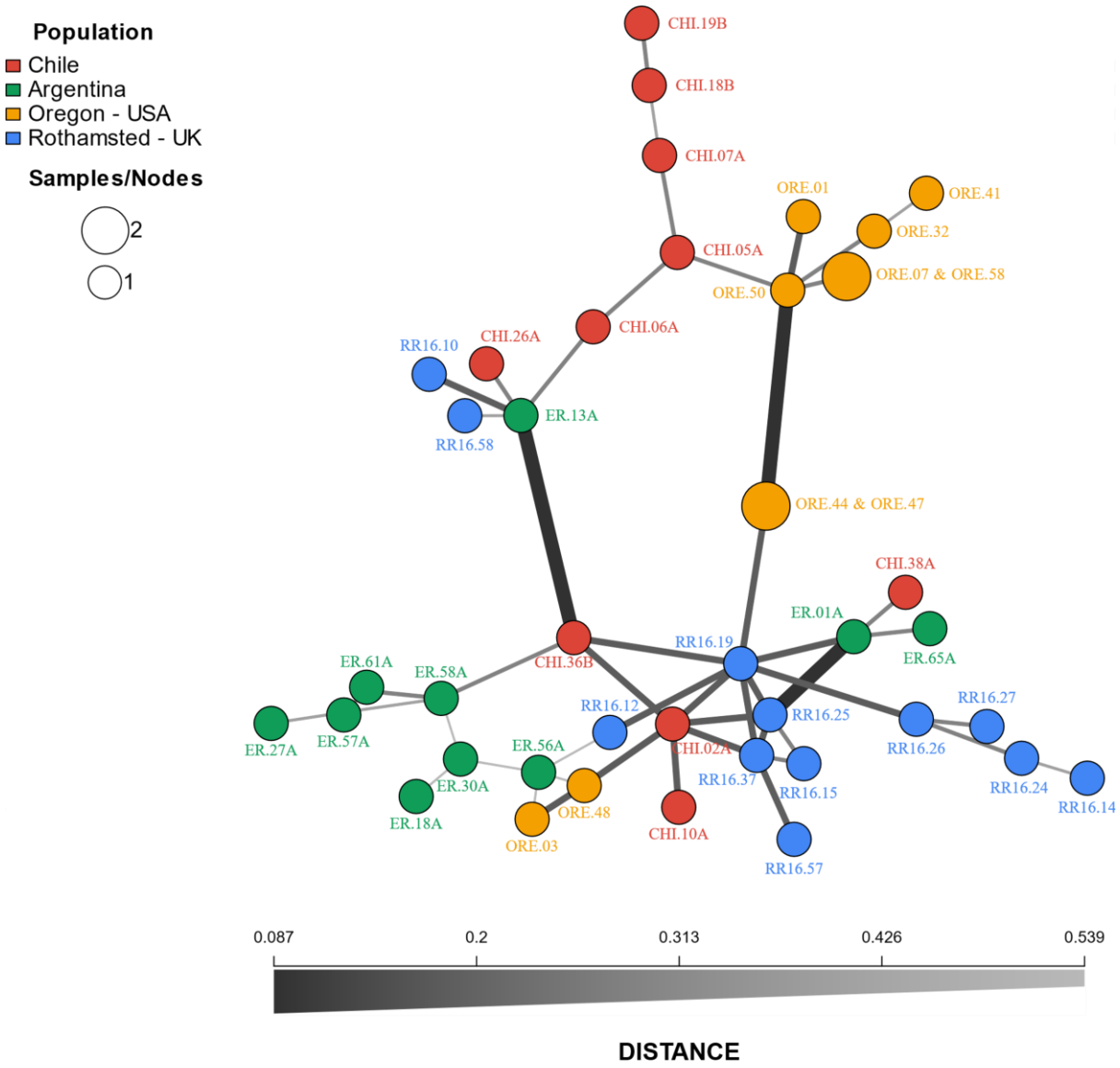

Figure 4.7.Minimum spanning network showing the relationships among the 40 multilocus genotypes (MLGs) of *Zymoseptoria tritici* with know CYP51 variant from Chile (CHI-Red), Argentina (ER-Green), Oregon-USA (ORE-Orange) and Rothamsted-UK (RR16-Blue). Distances and thickness of the lines between nodes are proportional to Nei's distance. Each node represents a different MLG and node sizes are proportional to the number of individuals. Loops within the network represent multiple paths between MLGs. The CYP51 variant associated with each MLG is shown in Table 4.6.

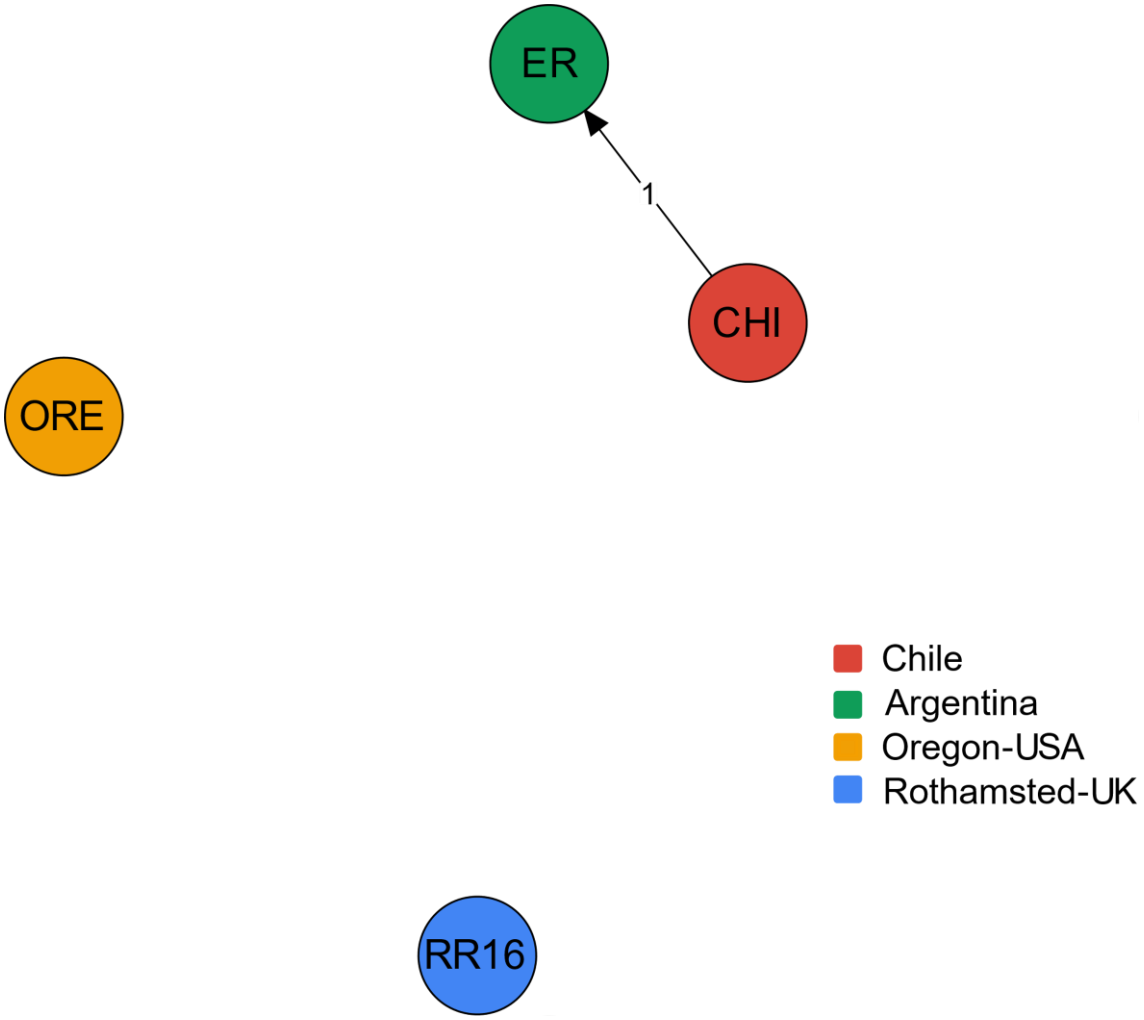

Figure 4.8 Directional relative migration network among *Zymoseptoria tritici* field populations from Chile (CHI-Red), Argentina (ER-Green), Oregon-USA (ORE-Orange) and Rothamsted-UK (RR16-Blue). Arrow indicates the direction of gene flow based on Jost's *D* measure with a bootstrap value of 1,000.

4.3.3 Reproductive mode

The low levels of estimated multilocus linkage disequilibrium mean that the null hypothesis for a random association of alleles between pairs of loci within each region was not rejected ($p > 0.05$ in each case). Low and no significant I_A (ranging from -0.01 to 0.16) and r_D values (ranging from -0.001 to 0.015) suggested free genetic recombination in the *Z. tritici* populations investigated (Table 4.7).

Table 4.7 Indexes of reproductive mode based on 12 microsatellite markers in *Zymoseptoria tritici* field populations from Chile, Argentina, Oregon-USA and Rothamsted-UK.

Population	n[1]	I_A[2]	r_D[3]	P[4]
Rothamsted-UK	46	0.16	0.015	0.283
Chile	45	0.05	0.005	0.289
Argentina	48	0.11	0.009	0.085
Oregon-USA	44	-0.01	-0.001	0.548

[1]Number of isolates.
[2]Index of association(I_A) between pairs of loci (Agapow & Burt, 2001).
[3]The standardized index of association corrected for the number of loci (Agapow & Burt, 2001).
[4]*P* values for I_A and r_D as determined by 1,000 randomizations.

4.4 Discussion

4.4.1 Genetic diversity

This study has focused on understanding the evolutionary process that led to the emergence of fungicide resistance in geographically isolated populations of *Z. tritici*. We tested the hypothesis that resistant alleles have arisen in Europe and then been introduced to naive populations in other continents. Therefore microsatellite markers were chosen to characterize the target populations because of their high rate of evolution (Linde *et al.*, 2010), making them sensitive markers to detecting recent population changes. Most of the SSR markers used in this study have already been proved to be highly efficient in characterizing global populations of *Z. tritici* (Gautier *et al.*, 2014). The set of markers used in the present study indeed were sufficiently powerful to discriminate almost all isolates as distinct MLGs and to characterize the different populations tested. High and similar levels of allelic and genotypic diversity were detected in field populations of *Z. tritici* from Rothamsted-UK, Chile, Argentina, and Oregon-USA sampled in 2016, with only one shared MLG among populations separated over 1,300 km. This is evidence that South American native populations have not been replaced by European isolates, but the CYP51 European genetic background was introgressed in those populations. We have found 100% of genotypic diversity in the Argentinian population sampled in the Entre-Rios region, in agreement with finds of Castillo *et al.* (2010) using ISSR markers in *Z. tritici* populations

sampled in the Buenos Aires Province. By contrast, other studies with *Z. tritici* isolates sampled in Argentina have found significantly lower levels of genotypic diversity when using RFLP as molecular markers (Cordo *et al.*, 2006; Jürgens *et al.*, 2006), but these are less sensitive than SSRs.

The frequency distribution of distinct MLGs among and within samples suggest that sexual reproduction is dominant in the studied populations. The observation of a random association of alleles and the low levels of multilocus linkage disequilibrium estimated in the present study are in accordance with the presence of the sexual stage reported for this pathogen in the countries where the populations were sampled (Madariaga, 1986; Scott *et al.*, 1988; Cordo *et al.*, 1990; DiLeone *et al.*, 1997; Hagerty, 2016). Therefore recurrent sexual recombination is likely to be the force driving the high genetic diversity observed in the present study (Zhan *et al.*, 2002, 2003).

4.4.2 Migration and population structure

Because *Z. tritici* populations were sampled in geographically distinct areas we tested the hypothesis of genetic differentiation between them. In recombining populations, the exchange of genetic information among populations results in a more homogeneous population structure. However, very low current gene flow is expected between the populations tested in the present study due to isolation by distance and geographical barriers, such as the Andes mountains or the Atlantic Ocean. Significant genetic differentiation was revealed between regions by both fixation indices and AMOVA. Likewise, genetic separation between regions was observed with both clustering analyses. The fact that most of the genetic variation was observed between individuals within the population agrees with other studies (McDonald *et al.*, 1995; Zhan *et al.*, 2003). Genetic structure has previously been reported among *Z. tritici* populations at large spatial scales, such as countries and continents (McDonald *et al.*, 1995; Drabešová *et al.*, 2013; Hartmann *et al.*, 2018). At intermediate spatial scales genetic structure has been observed among regional sub-collections within countries (Abrinbana *et al.*, 2010; Siah *et al.*, 2018) but not at small scales, such as between nearby fields and within single fields or single plants (Linde *et al.*, 2002; Siah *et al.*, 2018; Morais *et al.*, 2019). On the scale of separation of the populations

included in this study, mutation and genetic drift are driving forces of genetic differentiation, unopposed by gene flow. There will also be local adaptation of the pathogen to different climates, genetically different hosts, and cultural practices. These results reinforce the choice of microsatellites as a marker to detect recent demographic events. Previous studies have not observed or reported only little genetic structure in *Z. tritici* populations at regional and even global scales because less polymorphic, RFLP, markers were used (McDonald *et al.*, 1995; Linde *et al.*, 2002; Zhan *et al.*, 2003).

The lowest indices of genetic differentiation and most similar clustering were observed between the two South American populations. Moreover, they shared an MLG and divMigrate detected gene flow among these populations. It is not credible that wind-borne ascospores could be responsible for gene flow across the Andes mountains and large areas of non-wheat cultivation. Consequently, we supposed that commercial trade of wheat grain or exchange of infected plant material for breeding programs might account for some recent genotype flow between Argentina and Chile. Previous studies using sequence and microsatellite data of *Z. tritici* populations from Uruguay and Chile have concluded that they were genetically differentiated and hypothesized that they have been founded independently from each other, with higher similarity between Uruguayan and European populations while Chile appeared to be isolated (Banke & McDonald, 2005). This corroborates the previous findings of this work with the *CYP51* gene sequences. We conclude that independent migration events have introduced modern European CYP51 variants into Chile and Argentina/Uruguay. Nevertheless, some CYP51 variants were found on both sides of the Andes, which suggests the migration of a few individuals between those areas.

The four populations analysed in this study comprised *Z. tritici* isolates of admixed origin since all MLGs shared some ancestry. Additionally, distance-based and non-Bayesian clustering analyses suggested groupings of individuals independent of their origin. This spatial distribution of admixed MLG suggests strong genetic recombination starting from a recent common ancestor population. One hypothesis is that *Z. tritici* populations from North and South America were introduced to these continents from a

common and genetically diverse European source, resembling the population from Rothamsted-UK. The Bayesian clustering analysis showed that MLGs from Rothamsted had equivalent ancestry associated with all clusters, meanwhile, genotypes from the Americas were more associated to a specific cluster. Similarly, the minimum-spanning-network highlighted the European haplotypes in reticulated nodes while non-European haplotypes were located mostly on the tip clades. This supports the hypothesis that Rothamsted resembles the founder population from which the North and South American populations originated. The centre of origin for *Z. tritici* is assumed to be the middle east while the domestication of wheat and colonization of the New World dispersed the pathogen globally (Banke *et al.*, 2004). Banke & McDonald (2005), working with sequence data, reported that European populations were the source of most immigrants to South (Chile and Uruguay) and North American *Z. tritici* populations. In comparison, the same authors stated that populations from Mexico and Australia have received fewer migrants from European populations.

Z. tritici was reported in the countries involved in the present study many decades ago (Boerger, 1943; Gilchrist & Madariaga, 1980; Hagerty, 2016) though less than 600 years ago, since wheat is not native to the Americas. Hence, mutation and genetic drift over many generations of *Z. tritici* should have had time enough to generate population divergence. This hypothesis is in accordance with the significant genetic differentiation and genetic structure among populations observed in the present work. However, the admixed composition and clustering of MLGs sampled in geographically isolated areas, has one possible interpretation. A recent colonization event in South and North America with modern European haplotypes has happened. This hypothesis allows the CYP51 variants found in North and South American isolates to have emerged only once, probably in NW-Europe, as discussed in the previous chapter. The trade of contaminated seed (Brokenshire, 1975; Consolo *et al.*, 2009), could be responsible for the movement of a few individuals carrying resistant alleles into less adapted populations of *Z. tritici* recently exposed to fungicide selection pressure. In this scenario, high rates of genetic recombination would then be responsible for the introgression of the aforementioned resistant *CYP51* genes in the native genomic background. A reduction in effective population size due to a

selective sweep of resistant alleles would be the bottleneck to the fixation of azole-resistant alleles and an admixed genomic background resembling the European population.

Chapter 5: General Discussion

5.1 Main findings

The objective of this PhD study was to evaluate the status of fungicide resistance in South American field populations of *Z. tritici*. The experimental studies on this topic are described in three chapters. Chapter 2 describes the status of resistance to different classes of fungicides in populations sampled from two South American wheat-growing regions and compared it to populations sampled from other continents. Chapter 3 explores the molecular mechanisms responsible for fungicide resistance in populations sampled worldwide. Finally, in chapter 4 the differentiation between *Z. tritici* field populations from Europe, North and South America and the origins of azole, MBC and QoI resistance in those continents is discussed. This chapter also reiterates the main findings of the PhD project and its implications for the practical management of fungicide resistance and opportunities for future research on Septoria tritici blotch.

The most important finding from this research was that all South American populations of *Z. tritici* showed resistance to three different fungicide classes tested: MBCs, QoIs, and azoles. Moreover, azole resistance involves a similar spectrum of changes, largely in the same order, worldwide; and both gene flow and independent gain of mutations have contributed to the evolution of azole resistance in the Americas.

Fungicides have been used all over the world to control STB on wheat foliage for 3-4 decades. Loss of effectiveness to control STB due to resistance is well documented in Europe, North America, and Oceania. In NW-Europe STB is a major threat to wheat production, and extensive use of fungicides for its control has led to the emergence of fungicide resistance. The UK has experienced the loss of QoI fungicides for STB control in the early 2000s. This was soon followed by many other European countries, the USA and New Zealand (Lucas *et al.*, 2015). The new second generation of SDHI fungicides introduced in Europe to be used in cereals gave highly effective STB control, but resistant strains were quickly selected, putting the use of this class of fungicides at risk (Fraaije *et al.*, 2012; FRAC, 2019). The

effectiveness of azoles has eroded more slowly with the emergence and accumulation of multiple resistance mechanisms (Kirikyali *et al.*, 2017; Blake *et al.*, 2018; Kildea *et al.*, 2019), therefore, STB control nowadays relies on mixtures of this class with SDHIs. Chlorothalonil, a multisite with a low risk of fungicide resistance development, is also used in 2 or 3-way mixtures to improve disease control and as a resistance management strategy.

5.1.1 MBC and QoI resistances are widespread in field populations of *Zymoseptoria tritici* from different continents

The fact that MBC resistance is present in all four South American samples from both regions is not surprising. This class of fungicides was launched in the 1960s and represented a new era of chemical control of plant pathogens as the first of single-site systemic fungicides (Delp, 1995), and has thereafter been heavily used in arable crops. In the UK, for example, resistance to the MBC fungicides, due to a mutation in the target site encoding *β-tubulin* gene of *Z. tritici*, emerged in the mid-1980s (Griffin & Fisher, 1985; Lucas *et al.*, 2015). MBC fungicides were introduced in South America many decades ago and since then have been extensively used on wheat. Benomyl was first used in Uruguay in 1968 (Germán *et al.*, 1990). Carbendazim was tested for STB control in 1978 in Chile (Gilchrist & Madariaga, 1980). Such long term and extensive use of a single site fungicide put strong selective pressure for the emergence and rapid spread of resistant alleles. The frequency of the β-tubulin E198A alteration associated with carbendazim insensitivity ranged from 26% to 75% in the four South American *Z. tritici* populations, showing that the mutated allele is widespread. The reported loss of effectiveness of MBCs for STB control in South America (Ackermann, 2010) was probably linked to an increased frequency of E198A. This project also investigated other populations of *Z. tritici*, sampled from countries with less intensive use of fungicide. In North America, where benzimidazole was first used to STB control in the Willamette Valley of Oregon in 1985, resistance due to the β-tubulin E198A mutation emerged quickly (Hagerty, 2016). Currently, E198A frequency is as high as 68% in Oregon but only 29% in Michigan, where disease pressure and fungicide usage are lower. Previous reports on the onset of MBC resistance in STB from New Zealand could not be found in the literature, but this study detected a high frequency

of the E198A allele. In general, the β-tubulin E198A alteration is the global MBC resistance mechanism in *Z. tritici* populations. In all the regions sampled, the mutant allele could either have been introduced by European strains recombining with the local population or could have emerged *de novo* in an independent mutation event. Determining this was outside the scope of my work.

One potential problem in determining the degree of fungicide insensitivity on a global level is the small number of sampled populations and small population sample sizes. The sizes of individual samples here were around 50 isolates, so the standard error of frequency estimates is considerable. Therefore, results from sensitivity assays in this project do not represent the frequency of MBC resistance at the continental-level precisely, but they do indicate the intermediate and substantial frequencies of resistant strains. Further work should include a larger number of samples at a regional-scale to determine how distributed the resistant alleles are. However, MBC fungicides cause strong selection on E198A, and alleles can spread rapidly over long distances with ascospore movements. Moreover, there is no apparent fitness cost associated with this mutation in *Z. tritici*, and, consequently, its frequency only tends to increase. Thus, in a scenario of continuous use of MBC fungicides on wheat, this mutation will soon be fixed at high frequencies in South America and compounds within this class will not provide useful control of STB. In conclusion, this fungicide, if still in use, should be withdrawn from the wheat market in South America.

The loss of QoI effectiveness to control STB in South America has not been reported in the literature. However, resistance to QoI fungicides is widespread and at high frequencies in South America. Just as described above for MBCs, the molecular mechanism of QoI resistance in *Z. tritici* is the same all over the world, the mitochondrial cytochrome *b* G143A alteration leading to complete resistance. This project has not tested the relatedness of mitochondrial DNA between South American and European strains. Nonetheless, it has already been shown that G143A mutation has arisen in several different genomic backgrounds (Torriani *et al.*, 2009; Estep *et al.*, 2015), thus further analyses might verify the possibility of independent mutations for G143A in South America. Due to the absence of historical

collections, it would not be possible to define directly when resistance first emerged in this continent. Despite this, the extensive use of QoI fungicides to STB control in South America since the late 1990s seems to be directly associated with the emergence and widespread dispersal of the G143A mutation (Jobet *et al.*, 2013; INIA - Uruguay, 2017; INTA - Argentina, 2018).

Differences in the pattern of QoI resistance might help to understand the biology and management of a plant pathogen at the regional scale. Long distance dispersal of ascospores among fields results in a reduction of sensitivity at a region-wide level, regardless of whether all fields were treated with QoI fungicides or not. Thus, it is expected that *Z. tritici* populations east of the Andes will soon be completely insensitive to this class of fungicides. The same has been observed in a spatio-temporal analysis of azoxystrobin sensitivity in Oregon (Hagerty *et al.*, 2017). Another interesting observation is that QoI resistance is also present at high frequencies in New Zealand, where it has been reported since 2014 (Stewart *et al.*, 2014), but still absent in continental Australia and Tasmania (McDonald *et al.*, 2019) despite the use of QoIs in these two regions.

5.1.2 Migration and *de novo* emergence have contributed to the evolution of azole resistance worldwide

The most interesting finding from this research was the profile of azole resistance found around the globe. Azole fungicides have been a key component of control programs against *Z. tritici* since their introduction in the late 1970s, not only in Europe but also in other continents where this disease impacts wheat yield. In South America, azole fungicides have been used to control STB since the late 1980s (Galich *et al.*, 1990; Germán *et al.*, 1990; Mellado, 1990; Andrade, 1995) and increased during the 1990s with the introduction of new molecules within this class (Ackermann, 1996, 2010; Jobet *et al.*, 2013; Rosa *et al.*, 2013). Nowadays they are still used in mixtures with QoIs and SDHIs (Jobet *et al.*, 2013; INIA - Uruguay, 2017; INTA - Argentina, 2018). In contrast to the QoI and MBC fungicides, for which a single mutation confers full cross-resistance to all fungicides within these classes, mutations in the target site encoding *CYP51* gene confer only partial resistance to some azoles (Cools *et al.*, 2011).

CYP51 mutations in *Z. tritici* were first reported in the 1980s in Europe, followed by the sequential emergence of over 30 *CYP51* mutations in many different combinations (Leroux & Walker, 2011; Cools & Fraaije, 2013)

My sequencing data indicate that the reduced sensitivity in *Z. tritici* populations in South America is associated with CYP51 amino acid substitutions. Isolates from the four populations tested carry single CYP51 amino acid substitutions up to complex modern CYP51 variants with multiple alterations. The gradual accumulation of *CYP51* mutations is a response to the selection pressure imposed by the wide and diverse use of azoles over many years as described above. We identified different *CYP51* genetic backgrounds in South America. Basically, populations sampled East of the Andes mountains (Argentina and Uruguay) have two distinct ancestries. Strains with a highly reduced azole sensitivity carried modern European CYP51 variants, including the double amino acid deletion (ΔY459/G460). Analysis with molecular markers showed that European CYP51 encoding genes were introduced and introgressed in the South American genomic background in an environment of severe selection pressure. Strains that are more sensitive to azoles are carrying either the wild type CYP51 or have a few amino acid alterations. These CYP51 variants have the South American genetic background, implying a local emergence of azole resistance. On the other side of the Andes mountains, the population sampled in Chile also showed two distinct CYP51 ancestries. Contrasting with the other South American populations, Chile had both CYP51 lineages with European genetic background. This shows that several independent migration events have introduced European CYP51 encoding genes into South American populations of *Z. tritici*. Those genes have been introgressed into these populations' genomic backgrounds and are being selected, rather than the most complex variants evolving locally in the South American genetic background. The same conclusion can be assumed to apply to the population sampled in New Zealand, while Eastern European CYP51 haplotypes are a result of gene flow of resistant alleles in the direction from west to east in Europe and are easily accounted for by wind movement (Stammler *et al.*, 2008). However, the possibility that even the modern CYP51 variants, like those carrying the double amino acid deletion (ΔY459/G460), have evolved *de novo* in

different continents has to be considered and further investigated. McDonald *et al.* (2019) have suggested through more detailed whole-genome sequencing that azole resistance has emerged independently in Australia.

The azole fungicide resistance profile found in North American populations was rather different from South America. The limited number of amino acid alterations in CYP51 variants detected in North America might be due to a lack of gene flow from Europe. Although both North American populations showed CYP51 variants with the European genetic background, the less sensitive strains in Oregon revealed independent parallel resistance evolution to azoles. These strains carried mutations leading to amino acid alterations I381V and V136A, alterations with a high impact on azole binding, in the North American genetic background. Either way, azole resistance in this continent seems to be following the already known stepwise evolution observed in European populations.

5.2 Perspectives for disease management of Septoria tritici blotch

Results from this study indicate that MBC and QoI fungicides are unlikely to be effective against the majority of *Z. tritici* populations from across the globe. However, isolates from Michigan have not evolved resistance to MBC fungicides, as well as populations from Australia known to be sensitive to QoIs (McDonald *et al.*, 2019). Thus, although MBC and QoI resistance is likely to be widespread, both fungicide classes could be used in particular regions, provided the adoption of management tactics to avoid or delay the emergence of resistance, whether from local mutation or gene-flow. Further monitoring of *Z. tritici* populations will be needed to detect when effective disease control with these chemicals is lost.

From a practical perspective, the most worrying finding is that *Z. tritici* isolates from all continents had multiple CYP51 amino acid changes conferring reduced sensitivity across different azole molecules. The accumulation of CYP51 amino acid changes to produce azole resistance is a stepwise evolutionary process, thus it is reasonable to expect that South and North American strains will follow a similar evolutionary pathway to that of European isolates. As an example, the loss of two amino acids

(ΔY459/G460), required for the accumulation of other changes (Mullins *et al.*, 2011), is already present in some isolates from South America. The impact of the more complex combinations of alterations, including the presence of S524T, is well known in European populations of *Z. tritici* and has been conclusively shown by heterologous expression in *S. cerevisiae* (Cools *et al.*, 2010, 2011; Leroux & Walker, 2011).

From the foregoing results, slowing down the selection of further CYP51 amino acid alterations is essential. Agricultural practices should take into account resistance management tactics to prolong the effectiveness of azole compounds against STB in all countries involved in this study. In general, lower fungicide use leads to a reduction in selection pressure. In contrast, the increase in fungicide dose and number of applications of the same mode of action results in increased selection for less sensitive strains (Van den Bosch *et al.*, 2014), thereafter leading to fungicide loss of effectiveness. According to van den Bosch *et al.* (2011), lower dose rates are expected to reduce the selection pressure compared to higher doses. However, lower doses can also represent a reduction in disease control and therefore in wheat yield. The use of half doses in STB proved to be less selective for azole resistance compared to full doses but resulted in a reduction of disease control and wheat yield (Dooley *et al.*, 2016b). Reducing the number of fungicides sprays to once or twice a season also reduced the selection for more resistant strains compared to three applications, however, it compromised STB control and yield (Heick *et al.*, 2017). Although a mixture of different azoles showed better STB control than solo products, it increased the selection of strains less sensitive to both compounds (Dooley *et al.*, 2016b). Summarizing, a good level of control must be aimed at but with minimum use of any azole. This can be achieved by the alternation of compounds (Dooley *et al.*, 2016b), thus limiting the use of the same azole in a crop season. Also, if levels of Septoria tritici blotch are low, avoid the use of azoles to control high levels of other diseases like mildew and rusts, for which QoIs and mildewcides can be used.

Albeit reducing the number of applications will slow down the evolution of azole resistance, the continuous use of azoles will still select for *Z. tritici* with reduced sensitivity. The best practice to delay

the emergence or evolution of resistant strains is mixing or alternating different modes of action. Indeed, modelling studies found that mixing a single-site with a multisite fungicide delayed the emergence of resistance to the first in *Z. tritici* (Hobbelen *et al.*, 2014). This has been confirmed with practical experiments in which the best results in both yield and STB control were observed with mixtures and alterations of azoles with SDHIs and the multisite chlorothalonil. Moreover, this strategy selected less for CYP51 alterations (Heick *et al.*, 2017). This strategy is already recommended and widely adopted in the UK and other European countries (Agriculture and Horticulture Development Board, 2016). My study did not detect shifts in chlorothalonil sensitivity or resistant strains to SDHI fungicides in any population. Nonetheless, recently field isolates of *Z. tritici* carrying *Sdh* mutations conferring lower sensitivity to SDHI fungicides have been identified in Europe (Dooley *et al.*, 2016a; Kirikyali *et al.*, 2017; Rehfus *et al.*, 2018; FRAC, 2019).

The future of both azoles and chlorothalonil in Europe remains uncertain due to pesticide legislation (Hillocks, 2012; Goeritz *et al.*, 2014; Jess *et al.*, 2014). Therefore, the addition of new chemicals to the market is important to provide alternatives to growers. Fenpicoxamid, a Quinone-inside-Inhibitor (QiI) of a novel picolinamide class, is effective against *Z. tritici* and has no cross-resistance to QoI and azole fungicides (Owen *et al.*, 2017). However, *in vitro* selection experiments with *Saccharomyces cerevisiae* have already revealed amino acid substitutions in cytochrome bc1 residues N31, G37 and L198 that confer fenpicoxamid resistance and are conserved in *Z. tritici* (Young *et al.*, 2018). Metyltetraprole is a new QoI fungicide of the tetrazolinone chemical class and has shown to be efficient in the control of *Z. tritici* strains with G143A and F129L substitutions (Matsuzaki *et al.*, 2020). Mefentrifluconazole is an isopropanol-azole, a new chemical of the azole class. It is highly efficient in the control of azole insensitive *Z. tritici* strains carrying a range of different CYP51 variants. In addition, it is in conformity to new European regulatory standards on endocrine disruption effects (Bryson *et al.*, 2018). *In vitro* sensitivity tests have shown signs of cross-resistance between mefentrifluconazole and tebuconazole in field isolates of *Z. tritici*, with lower sensitivity observed in strains with MDR phenotype (B. Fraaije, *personal communication*).

IPM (Integrated Pest Management) practices combining all the above to other management strategies, such as the use of resistant varieties and fungicide spraying based on disease forecasting, would further reduce fungicide input and consequently slow down resistance development. In South America, resistance management can be achieved by limiting the exposure of the pathogen to fungicides through agricultural practices that have proven to reduce the early incidence of inoculum, i.e. stubble management and manipulation of the sowing date (Caglevic, 1982; Andrade & Contreras, 2007; Ackermann, 2010).

5.3 Future work to study the development of fungicide resistance

In summary, the three experimental chapters of this thesis contribute to the current knowledge about fungicide sensitivity in South American *Z. tritici* populations and indicate that this is a major concern all over the globe. It has also highlighted topics for further work, in particular: characterize different inserts in the promoter regions of *CYP51* and *MFS1*; elucidate molecular mechanisms underlying reduced fluopyram sensitivity in South American isolates; conduct selection experiments to test *in vitro* experimental evolution of resistance with azole and SDHI fungicides in South and North American strains; estimate fitness costs associated to azole resistance with South and North American isolates; carry on regional surveys to assess the widespread of fungicide resistant strains in South America; investigate the ways European *Z. tritici* strains have migrated into South American populations to mitigate the introgression of fungicide resistance alleles, and research on IPM tactics specific for each region to reduce the incidence of disease and consequently delay selection for fungicide resistance.

References

Abrinbana M, Mozafari J, Shams-bakhsh M, Mehrabi R, 2010. Genetic structure of *Mycosphaerella graminicola* populations in Iran. *Plant Pathology* **59**, 829–838.

Ackermann MD de, 1996. Mancha foliar del trigo causada por *Mycosphaerella graminicola* (Fuckel) Schroeter estado perfecto de *Septoria tritici* Rob. ex Desm. In: Díaz M, ed. *Manejo de enfermedades en cereales de invierno y pasturas*. Montevideo - Uruguay: INIA Serie Actividades de Difusión; 74, 43–62.

Ackermann MD de, 2010. Mancha de la hoja o Septoriosis del trigo. In: *Seminario de Actualización: Manejo de Enfermedades en Trigo y Cebada*. La Estanzuela, Colonia (Uruguay): INIA Serie Actividades de Difusión; 618, 63–80.

Agapow P-M, Burt A, 2001. Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes* **1**, 101–102.

Agriculture and Horticulture Development Board, 2016. Wheat disease management guide. *AHDB Cereals & Oilseeds*, 28.

Allen EA, 1991. Appressorium formation in response to topographical signals by 27 rust species. *Phytopathology* **81**, 323.

Andrade O, 1990. Importancia de *Septoria tritici* en Chile y progresos en los trabajos realizados. In: Kohli MM, Beuningen LT van, eds. *Conferencia regional sobre la septoriosis del trigo*. Mexico, D.F: CIMMYT, 229–239.

Andrade O, 1995. Mancha de la hoja del trigo *Mycosphaerella graminicola* en la zona sur de Chile. In: Gilchrist SL, Ginkel M van, McNab A, Kema GHJ, eds. *Septoria tritici Workshop*. Mexico, D.F: CIMMYT, 84–94.

Andrade O, Contreras E, 2007. La mancha foliar o Septoriosis de la hoja del trigo. *Informativo INIA Carillanca*, 2.

Arenas M, Posada D, 2010. The effect of recombination on the reconstruction of ancestral sequences. *Genetics* **184**, 1133–1139.

Avenot HF, Michailides TJ, 2010. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Protection* **29**, 643–651.

Banke S, McDonald BA, 2005. Migration patterns among global populations of the pathogenic fungus *Mycosphaerella graminicola*. *Molecular Ecology* **14**, 1881–1896.

Banke S, Peschon A, McDonald BA, 2004. Phylogenetic analysis of globally distributed *Mycosphaerella graminicola* populations based on three DNA sequence loci. *Fungal Genetics and Biology* **41**, 226–238.

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B, 2002. The strobilurin fungicides. *Pest Management Science* **58**, 649–662.

Berg D, Plempel M, 1998. *Sterol biosynthesis inhibitors* (D Berg, M Plempel, Eds,). Chichester: VCH.

Birchmore RJ, Williams RJ, Russell PE, Lagouarde P, 1996. A baseline for the sensitivity of *Botrytis cinerea* to pyrimethanil. In: *Brighton Crop Protection Conference - Pests and Diseases*. AgrEvo UK Limited, Chesterford Park, Saffron Walden, Essex (United Kingdom), 713–718.

Blake JJ, Gosling P, Fraaije BA *et al.*, 2018. Changes in field dose-response curves for demethylation inhibitor (DMI) and quinone outside inhibitor (QoI) fungicides against *Zymoseptoria tritici*, related to laboratory sensitivity phenotyping and genotyping assays. *Pest Management Science* **74**, 302–313.

Boeger JM, Chen RS, McDonald BA, 1993. Gene flow between geographic populations of *Mycosphaerella graminicola* (anamorph *Septoria tritici*) detected with Restriction Fragment Length Polymorphism Markers. *Phytopathology* **83**, 1148–1154.

Boerger A, 1943. *Investigaciones agronómicas*. Montevideo: Casa a Barreiro y Ramos SA.

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

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.

Brading P a, Verstappen ECP, Kema GHJ, Brown JKM, 2002. A gene-for-gene relationship between wheat and *Mycosphaerella graminicola*, the Septoria tritici blotch pathogen. *Phytopathology* **92**, 439–445.

Brent KJ, Hollomon DW, 2007. *Fungicide Resistance in Plant Management: How can it be managed?* Fungicide Resistance Action Committee 2007.

Brokenshire T, 1975. Wheat seed infection by *Septoria tritici*. *Transactions of the British Mycological Society* **64**, 331–334.

Brown JKM, Chartrain L, Lasserre-Zuber P, Saintenac C, 2015. Genetics of resistance to *Zymoseptoria tritici* and applications to wheat breeding. *Fungal Genetics and Biology* **79**, 33–41.

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**, 305–316.

Bryson RJ, Stammler G, Huff A, Strobel D, Meyer L, Moronval MH, 2018. Mefentrifluconazole - the first Isopropanol-azole fungicide for the control of *Zymospetoria tritici* including field isolates with known complex CYP51 haplotypes. In: *12e Conférence Internationale sur les Maladies des Plantes*. Tours, France: Végéphyl, 222–231.

Caglevic M, 1982. La septoriosis de la hoja del trigo. *IP La Platina* **14**, 25–29.

Campos M, 2008. Variedades y modelos generales de produccion del movimento CREA. *Produccion de Trigo*, 73–118.

Castillo N, Cordo C, Simón MR, 2010. Molecular variability among isolates of *Mycosphaerella graminicola*, the causal agent of Septoria tritici blotch, in Argentina. *Phytoparasitica* **38**, 379–389.

El Chartouni L, Tisserant B, Siah A *et al.*, 2012. Evolution of *Mycosphaerella graminicola* at the wheat leaf and field levels. *Phytopathologia Mediterranea* **51**, 332–339.

Chassot C, Hugelshofer U, Sierotzki H, Gisi U, 2008. Sensitivity of CYP51 genotypes to DMI fungicides in *Mycosphaerella graminicola*. In: Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE, Lyr H, eds. *Modern Fungicides and Antifungal Compounds V*. Braunschweig, Germany: Deutsche Phytomedizinische Gesellschaft, 129–136.

Chen RS, McDonald BA, 1996. Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. *Genetics* **142**, 1119–27.

Clement M, Snell Q, Walker P, Posada D, Randall K, 2002. TCS: Estimating gene genealogies. In: *International parallel and distributed processing symposium*. Fort Lauderdale, FL: IEEE Computer Society, 184.

Consolo VF, Albani CM, Berón CM, Salerno GL, Cordo CA, 2009. A conventional PCR technique to detect *Septoria tritici* in wheat seeds. *Australasian Plant Pathology* **38**, 222.

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

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

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

Cools HJ, Mullins JGL, Fraaije BA *et al.*, 2011. Impact of recently emerged sterol 14α-demethylase (CYP51) variants of *Mycosphaerella graminicola* on azole fungicide sensitivity. *Applied and Environmental Microbiology* **77**, 3830–3837.

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**, 2866–2872.

Cordo CA, 2010. Epidemiological studies on Septoria leaf blotch of wheat in Argentina. In: Arya A, Perelló AE, eds. *Management of fungal plant pathogens*. Wallingford: CABI, 291–308.

Cordo CA, Linde CC, Zhan J, Mcdonald B, 2006. Genotypic diversity of the wheat leaf blotch pathogen (*Septoria tritici*) in Buenos Aires Province. *Boletin de la Sociedad Argentina de Botanica* **41**, 293–305.

Cordo CA, Perelló AE, Alippi HE, Arriaga HO, 1990. Presence of *Mycosphaerella graminicola* (Fuckel) Schroeter teleomorph *Septoria tritici* Rob. ex Desm. in maturity wheat of Argentina. *Revista de la Facultad de Agronomía* **66**–**67**, 49–55.

Cordo CA, Simon MR, Perello AE, Alippi HE, 1999. Spore dispersal of leaf blotch pathogens of wheat (*Mycosphaerella graminicola* and *Septoria tritici*). In: Ginkel M van, Krupinsky J, eds. *Septoria and Stagonospora Diseases of Cereals: A Compilation of Global Research*. Mexico City, Mexico: CIMMYT, 98–101.

Cordo CA, Trofino C, Stocco MC, Monaco CI, Kripelz NI, Balatti P, 2017. Cambio en la sensibilidad al fungicida tebuconazol en las poblaciones de *Zymoseptoria tritici*, patógeno de la septoriosis de la hoja del trigo. In: *Cuarto Congreso International Cientifico Y Tecnologico De La Provincia De Buenos Aires*. Buenos Aires, 14.

Cromey MG, Braithwaite M, Alexander BJR, Ganev S, Cookson TR, 2000. Control of speckled leaf blotch of wheat with fungicides. *New Zealand Plant Protection* **53**, 103–108.

Davidse LC, Ishii H, 1995. Biochemical and molecular aspects of action of benzimidazoles, N-

phenylcarbamates and N-phenylformamidoximes and the mechanism of resistance to these compounds in fungi. In: Lyr H, ed. *Modern Selective Fungicides*. New York: Gustav Fischer, 595.

Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE, Lyr H (Eds.), 2010. Modern fungicides and antifungal compounds VI. In: *16th International Reinhardsbrunn Symposium*. Friedrichroda, Germany: Spectrum Phytomedizin, 438.

Delp C, 1995. Benzimidazole and related fungicides. In: Lyr H, ed. *Modern Selective Fungicides*. New York: Gustav Fischer, 595.

Delp C, J., 1988. *Fungicide Resistance in North America*. Minnesota: American Phytopathological Society.

Delport W, Poon AFY, Frost SDW, Kosakovsky Pond SL, 2010. Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. *Bioinformatics* **26**, 2455–2457.

DiLeone JA, Karow RS, Mundt CC, Coakley SM, 1997. Biology and control of the septoria diseases of winter wheat in western Oregon. *Crop Science Report 109. Oregon State University. Agricultural Experiment Station.*, 21.

Dooley H, 2015. Fungicide resistance management tactics: impacts on *Zymoseptoria tritici* populations. University of Reading.

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

Dooley H, Shaw MW, Spink J, Kildea S, 2016b. Effect of azole fungicide mixtures, alternations and dose on azole sensitivity in the wheat pathogen *Zymoseptoria tritici*. *Plant Pathology* **65**, 124–136.

Dooley H, Shaw MW, Spink J, Kildea S, 2016c. The effect of succinate dehydrogenase inhibitor/azole mixtures on selection of *Zymoseptoria tritici* isolates with reduced sensitivity. *Pest Management Science* **72**, 1150–1159.

Drabešová J, Ryšánek P, Brunner P, McDonald BA, 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**, 211–224.

Dubos T, Pasquali M, Pogoda F, Casanova A, Hoffmann L, Beyer M, 2013. Differences between the succinate dehydrogenase sequences of isopyrazam sensitive *Zymoseptoria tritici* and insensitive *Fusarium graminearum* strains. *Pesticide Biochemistry and Physiology* **105**, 28–35.

Duncan KE, Howard RJ, 2000. Cytological analysis of wheat infection by the leaf blotch pathogen *Mycosphaerella graminicola*. *Mycological Research* **104**, 1074–1082.

Earl DA, von Holdt BM, 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* **4**, 359–361.

Eriksen L, Shaw MW, Østergård H, 2001. A model of the effect of pseudothecia on genetic recombination and epidemic development in populations of *Mycosphaerella graminicola*. *Phytopathology* **91**, 240–248.

Estep LK, Torriani SFF, Zala M *et al.*, 2015. Emergence and early evolution of fungicide resistance in North American populations of *Zymoseptoria tritici*. *Plant Pathology* **64**, 961–971.

Estep LK, Zala M, Anderson NP *et al.*, 2013. First report of resistance to QoI fungicides in North American populations of *Zymoseptoria tritici*, causal agent of Septoria tritici blotch of wheat. *Plant Disease* **97**, 1511–1511.

Evanno G, Regnaut S, Goudet J, 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology* **14**, 2611–2620.

Excoffier L, Lischer HEL, 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**, 564–567.

Eyal Z, 1971. The kinetics of pycnidiospore liberation in *Septoria tritici*. *Canadian Journal of Botany* **49**, 1095–1099.

Eyal Z, Amiri Z, Wahl I, 1973. Physiological specialization of *Septoria tritici*. *Phytopathology* **63**, 1087–1091.

Eyal Z, Scharen AL, Prescott JM, van Ginkel M, 1987. *The Septoria diseases of wheat: Concepts and methods of disease management*. Mexico, D.F: CIMMYT.

FAO, 2019. *Food Outlook - Biannual Report on Global Food Markets*. Rome.

FAR, 2018. *Cropping Strategies - a summary of recent research findings: Cereal Disease Management (Revised 2018)*. Christchurch, New Zealand.

Fleitas MC, Gerard GS, Simón MR, 2016. Eficacia residual de fungicidas sobre la roya de la hoja del trigo y su efecto sobre componentes del rendimiento y porcentaje de proteínas en grano. *FAVE Sección Ciencias Agrarias* **14**, 69–84.

Flor HH, 1971. Current status of the Gene-For-Gene concept. *Annual Review of Phytopathology* **9**, 275–296.

Fones HN, Eyles CJ, Kay W, Cowper J, Gurr SJ, 2017. A role for random, humidity-dependent epiphytic growth prior to invasion of wheat by *Zymoseptoria tritici*. *Fungal Genetics and Biology* **106**, 51–60.

Fones H, Gurr S, 2015. The impact of Septoria tritici blotch disease on wheat: An EU perspective. *Fungal Genetics and Biology* **79**, 3–7.

Fraaije BA, Bayon C, Atkins S, Cools HJ, Lucas JA, Fraaije MW, 2012. Risk assessment studies on succinate dehydrogenase inhibitors, the new weapons in the battle to control Septoria leaf blotch in wheat. *Molecular Plant Pathology* **13**, 263–275.

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**, 933–941.

Fraaije BA, Cools HJ, Kim S-H, Motteram J, Clark WS, Lucas JA, 2007. A novel substitution I381V in the sterol 14α-demethylase (CYP51) of *Mycosphaerella graminicola* is differentially selected by azole fungicides. *Molecular Plant Pathology* **8**, 245–254.

Fraaije BA, Lucas JA, Clark WS, Burnett FJ, 2003. QoI resistance development in populations of cereal pathogens in the UK. In: *British Crop Protection Council International Congress - Crop Science & Technology*. Alton, Hampshire, UK, 689–694.

FRAC, 2006. Mutations associated with QoI-resistance. *Fungicide Resistance Action Committee (FRAC) - QoI Working Group*, 2.

FRAC, 2016. Protocol of the discussions and use recommendations of the SDHI Working Group of the Fungicide Resistance Action Committee (FRAC). *Fungicide Resistance Action Committee (FRAC) - SDHI Working Group*, 14.

FRAC, 2017. FRAC Code List 2017: Fungicides sorted by mode of action. *Fungicide Resistance Action Committee (FRAC)*, 12.

FRAC, 2019. Protocol of the discussions and use recommendations of the SDHI Working Group of the Fungicide Resistance Action Committee (FRAC). *Fungicide Resistance Action Committee (FRAC) - SDHI Working Group*, 18.

FRAG-UK, 2015. FRAG-UK: statement on SDHI fungicides and resistance risk in cereals. *AHDB Website*.

Fritz A, Scholz MF, 2019. K-Sample rank tests and their combinations. *Package K-Samples - CRAN*, 34.

Galdames RG, 2013. *Principales enfermedades del trigo en el borde costero de la región de la Araucanía* (I de IACR de I Carillanca, Ed,). Temuco - Chile: EL-32 Boletin INIA - Instituto de Investigaciones Agropecuarias no. 281.

Galich A, Galich MV de, Legasa A, 1990. Control quimico de *Septoria tritici*. In: Kohli MM,, Beuningen LT van, eds. *Conferencia regional sobre la septoriosis del trigo*. Mexico, D.F: CIMMYT, 209–215.

Garnault M, Duplaix C, Leroux P *et al.*, 2019. Spatiotemporal dynamics of fungicide resistance in the wheat pathogen *Zymoseptoria tritici* in France. *Pest Management Science* **75**, 1794–1807.

Gautier A, Marcel TC, Confais J *et al.*, 2014. Development of a rapid multiplex SSR genotyping method to study populations of the fungal plant pathogen *Zymoseptoria tritici*. *BMC Research Notes* **7**, 373.

Germán S, Barcellos A, Chaves M, Kohli M, Campos P, de Viedma L, 2007. The situation of common wheat rusts in the Southern Cone of America and perspectives for control. *Australian Journal of Agricultural Research* **58**, 620.

Germán S, Perea C, Ackermann MD de, 1990. Importancia de *Septoria tritici* en Uruguay y avance en los trabajos realizados. In: Kohli MM, Beuningen LT van, eds. *Conferencia regional sobre la septoriosis del trigo*. Mexico, D.F: CIMMYT, 64–79.

Gilchrist L, Madariaga R, 1980. Antecedentes sobre septoriosis (*Septoria tritici* Desm.) en Chile. In: *Primera Reunión sobre Septoria tritici*. Estanzuela, Uruguay,.

Gisi U, Sierotzki H, Cook A, McCaffery A, 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Management Science* **58**, 859–867.

Goeritz I, Atorf C, Whalley P, Seymour P, Klein M, Schlechtriem C, 2014. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009. *Journal of the science of food and agriculture* **94**, 621–630.

Goudet J, 1995. FSTAT (Version 1.2): A computer program to calculate F-Statistics. *Journal of Heredity* **86**, 485–486.

Griffin MJ, Fisher N, 1985. Laboratory studies on benzimidazole resistance in *Septoria tritici*. *EPPO Bulletin* **15**, 505–511.

Grünwald NJ, Goodwin SB, Milgroom MG, Fry WE, 2003. Analysis of genotypic diversity data for populations of microorganisms. *Phytopathology* **93**, 738–746.

Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O, 2010. New algorithms and methods to estimate Maximum-Likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology* **59**, 307–321.

Hagerty CH, 2016. Dynamics of *Zymoseptoria tritici* fungicide resistance in the Willamette Valley of Oregon. Oregon State University.

Hagerty CH, Anderson NP, Mundt CC, 2017. Temporal dynamics and spatial variation of azoxystrobin and propiconazole resistance in *Zymoseptoria tritici*: A hierarchical survey of commercial winter wheat fields in the Willamette Valley, Oregon. *Phytopathology* **107**, 345–352.

Hartmann FE, McDonald BA, Croll D, 2018. Genome-wide evidence for divergent selection between populations of a major agricultural pathogen. *Molecular Ecology* **27**, 2725–2741.

Hawkins NJ, Bass C, Dixon A, Neve P, 2019. The evolutionary origins of pesticide resistance. *Biological Reviews* **94**, 135–155.

Hayes LE, Sackett KE, Anderson NP, Flowers MD, Mundt CC, 2016. Evidence of selection for fungicide resistance in *Zymoseptoria tritici* populations on wheat in Western Oregon. *Plant Disease* **100**, 483–489.

Heick TM, Justesen AF, Jørgensen LN, 2017. Anti-resistance strategies for fungicides against wheat pathogen *Zymoseptoria tritici* with focus on DMI fungicides. *Crop Protection* **99**, 108–117.

Hillocks RJ, 2012. Farming with fewer pesticides: EU pesticide review and resulting challenges for UK agriculture. *Crop Protection* **31**, 85–93.

Hobbelen PHF, Paveley ND, van den Bosch F, 2014. The emergence of resistance to fungicides (J Sturtevant, Ed,). *PLoS ONE* **9**, e91910.

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

INIA - Uruguay, 2017. Actualización sanitaria para trigo y aspectos a tener en cuenta para el manejo. *2da Comunicación*, 4.

INTA - Argentina, 2018. Trigo 2018 - Informe de actualización técnico en línea N^{o} 10. *Trigo Actualización 2018*, 191.

Ishii H, 1992. Target site of tubulin-binding fungicides. In: Koller W, ed. *Target sites of fungicides action*. London: CRC Press, 328.

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**, 1646–1654.

Jobet CF, Espinoza NN, Campillo RR, Madariaga RB, 2013. Recomendaciones técnicas - Cultivo de trigo en la región de la Araucanía. *INIA Informativo N^{o} 63*, 4.

Jombart T, Ahmed I, 2011. adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. *Bioinformatics* **27**, 3070–3071.

Jombart T, Devillard S, Balloux F, 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics* **11**, 94.

Jost L, 2008. GST and its relatives do not measure differentiation. *Molecular Ecology* **17**, 4015–4026.

Jürgens T, Linde CC, McDonald BA, 2006. Genetic structure of *Mycosphaerella graminicola* populations from Iran, Argentina and Australia. *European Journal of Plant Pathology* **115**, 223–233.

Kamvar ZN, Brooks JC, Grünwald NJ, 2015. Novel R tools for analysis of genome-wide population genetic data with emphasis on clonality. *Frontiers in Genetics* **6**.

Kamvar ZN, Tabima JF, Grünwald NJ, 2014. Poppr : an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* **2**, e281.

Katoh K, Standley DM, 2013. MAFFT multiple sequence alignment software Version 7: Improvements in performance and usability. *Molecular Biology and Evolution* **30**, 772–780.

Kema GHJ, 1990. Control quimico contra las especies de Septoria que atacan al trigo en Europa. In: Kohli MM, Van Beuningen LT, eds. *Conferencia regional sobre la septoriosis del trigo*. Mexico, D.F: CIMMYT, 240–251.

Kema G, 1996. Histology of the pathogenesis of *Mycosphaerella graminicola* in wheat. *Phytopathology* **86**, 777.

Kema GHJ, Mirzadi Gohari A, Aouini L *et al.*, 2018. Stress and sexual reproduction affect the dynamics of the wheat pathogen effector AvrStb6 and strobilurin resistance. *Nature Genetics* **50**, 375–380.

Keon JPR, White GA, Hargreaves JA, 1991. Isolation, characterization and sequence of a gene conferring resistance to the systemic fungicide carboxin from the maize smut pathogen, *Ustilago maydis*. *Current genetics* **19**, 475–481.

Kildea S, Dunne B, Mullins E, Cooke LR, Mercer PC, O'Sullivan E, 2010. Pyraclostrobin reduces germ tube growth of QoI-resistant *Mycosphaerella graminicola* pycnidiospores and the severity of

Septoria tritici blotch on winter wheat. *Plant Pathology* **59**, 1091–1098.

Kildea S, Marten-Heick T, Grant J, Mehenni-Ciz J, Dooley H, 2019. A combination of target-site alterations, overexpression and enhanced efflux activity contribute to reduced azole sensitivity present in the Irish *Zymoseptoria tritici* population. *European Journal of Plant Pathology* **154**, 529–540.

Kirikyali N, Diez P, Luo J, Hawkins N, Fraaije B, 2017. Azole and SDHI sensitivity of *Zymoseptoria tritici* field populations sampled in France, Germany and United Kingdom during 2015. In: Deising HB, Fraaije B, Mehl A, Oerke EC, Sierotzki H, Stammler G, eds. *Modern Fungicides and Antifungal Compounds VIII*. Friedrichroda: DPG Spectrum Phytomedizin, 153–158.

Kohli MM, Ackermann MD de, 1998. Evaluating southern cone wheat germplasm for spot blotch and tan spot. In: *Helminthosporium Blights of Wheat: Spot Blotch and Tan Spot*. CIMMYT, 230–240.

Koller W, 1992. Antifungal agents with target sites in sterol functions and biosynthesis. In: Koller W, ed. *Target sites of fungicide action*. London: CRC Press, 328.

Kosakovsky Pond SL, Posada D, Gravenor MB, Woelk CH, Frost SDW, 2006. Automated phylogenetic detection of recombination using a genetic algorithm. *Molecular Biology and Evolution* **23**, 1891–1901.

Kottek M, Grieser J, Beck C, Rudolf B, Rubel F, 2006. World Map of the Köppen-Geiger climate classification updated. *Meteorologische Zeitschrift* **15**, 259–263.

Kraan G, Nisi JE, 1993. Septoriosis del trigo en la Republica Argentina. Situacion del cultivo frente a la enfermedad. In: Gilchrist LS, van Ginkel M, McNab A, Kema GHJ, eds. *Septoria tritici Workshop*. Mexico City, Mexico: CIMMYT, 1–8.

Kuck KH, Scheinpglug H, Pontzen R, 1995. DMI fungicides. In: Lyr H, ed. *Modern Selective Fungicides*. New York: Gustav Fischer, 595.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K, 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms (FU Battistuzzi, Ed,). *Molecular Biology and Evolution* **35**, 1547–1549.

Kwok IMY, 1993. The biochemical mode of action of newer azole fungicides. University of Bristol.

Kwok IMY, Loeffler RT, 1993. The biochemical mode of action of some newer azole fungicides. *Pesticide Science* **39**, 1–11.

Lendenmann MH, Croll D, McDonald BA, 2015. QTL mapping of fungicide sensitivity reveals novel genes and pleiotropy with melanization in the pathogen *Zymoseptoria tritici*. *Fungal Genetics and*

Biology **80**, 53–67.

Leroux P, Albertini C, Gautier A, Gredt M, Walker A-S, 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**, 688–698.

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

Linde CC, Liles JA, Thrall PH, 2010. Expansion of genetic diversity in randomly mating founder populations of *Alternaria brassicicola* infecting *Cakile maritima* in Australia. *Applied and Environmental Microbiology* **76**, 1946–1954.

Linde CC, Zhan J, McDonald BA, 2002. Population structure of *Mycosphaerella graminicola*: From lesions to continents. *Phytopathology* **92**, 946–955.

Lucas JA, Fraaije BA, 2007. QoI resistance in *Mycosphaerella graminicola*: What have we learned so far? In: Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE, Lyr H, eds. *Modern Fungicides and Antifungal Compounds V - 15th International Reinhardsbrunn Symposium*. Friedrichroda, Germany: Spectrum Phytomedizin, 71–77.

Lucas JA, Fraaije BA, 2008. The final straw: crop archives and the recent history of cereal pathosystems. In: Jellis G, Edwards C, eds. *BSPP Presidential Meeting, Cereal Pathosystems*. London: British Society for Plant Pathology, 103–115.

Lucas JA, Hawkins NJ, Fraaije BA, 2015. The evolution of fungicide resistance. In: *Advances in Applied Microbiology*. 29–92.

Ma Z, Michailides TJ, 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Protection* **24**, 853–863.

Madariaga RB, 1986. The occurrence in Chile of *Mycosphaerella graminicola* (Fuckel) Schroeter, teleomorph of *Septoria tritici* Rob. ex. Dem. *Agricultura Tecnica* **46**, 209–211.

Madariaga R, 2011. Lancero INIA [BEZ/4/II.5072/Y54//NOR10B/3/MD/IB] the perfect alternative wheat cv. for Chile but susceptible to the *Mycosphaerella graminicola – Septoria tritici* pathosystem. In: Duveiller E, Singh P, eds. *8th International Symposium on Mycosphaerella and Stagonospora Diseases of Cereals, CIMMYT*. Mexico City,.

Madariaga B. R, Scharen AL, 1985. Septoria tritici blotch in Chilean wild oat. *Plant Disease* **69**, 126–127.

MAGYP, 2019. Serie de Tiempo Estimación de Trigo Pan - Argentina. *Datos Abiertos Agroindustria*.

Marroni M V, Viljanen-Rollinson SLH, Butler RC, Deng Y, 2006. Fungicide timing for the control of Septoria tritici blotch of wheat. *New Zealand Plant Protection* **59**, 160–165.

Matsuzaki Y, Kiguchi S, Suemoto H, Iwahashi F, 2020. Antifungal activity of metyltetraprole against the existing QoI-resistant isolates of various plant pathogenic fungi. *Pest Management Science* **76**, 1743–1750.

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**, 259–266.

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**, 715–725.

McDonald BA, Linde C, 2002. The population genetics of plant pathogens and breeding strategies for durable resistance. In: *Euphytica*. 163–180.

McDonald BA, Pettway RE, Chen RS, Boeger JM, Martinez JP, 1995. The population genetics of *Septoria tritici* (teleomorph *Mycosphaerella graminicola*). *Canadian Journal of Botany* **73**, 292–301.

McDonald MC, Renkin M, Spackman M *et al.*, 2019. Rapid parallel evolution of azole fungicide resistance in Australian populations of the wheat pathogen *Zymoseptoria tritici* (C Vieille, Ed,). *Applied and environmental microbiology* **85**.

Meirmans PG, Hedrick PW, 2011. Assessing population structure: FST and related measures. *Molecular Ecology Resources* **11**, 5–18.

Mellado M, 1990. Septoriosis del trigo en el Centro Sur de Chile. In: Kohli MM, Beuningen LT van, eds. *Conferencia regional sobre la septoriosis del trigo*. Mexico, D.F: CIMMYT, 15–34.

Mikaberidze A, Paveley N, Bonhoeffer S, van den Bosch F, 2017. Emergence of resistance to fungicides: The role of fungicide dose. *Phytopathology* **107**, 545–560.

Milgroom MG, 2017a. Migration and population structure. In: Milgroom MG, ed. *Population Biology of Plant Pathogens: Genetics, Ecology, and Evolution*. The American Phytopathological Society, 119–146.

Milgroom MG, 2017b. *Population biology of plant pathogens: genetics, ecology, and evolution*. The American Phytopathological Society.

Morais D, Duplaix C, Sache I, Laval V, Suffert F, Walker A-S, 2019. Overall stability in the genetic structure of a *Zymoseptoria tritici* population from epidemic to interepidemic stages at a small spatial scale. *European Journal of Plant Pathology* **154**, 423–436.

Moreno MV, Perelló AE, 2010. Occurrence of *Pyrenophora tritici-repentis* causing tan spot in Argentina. In: *Management of fungal plant pathogens*. Wallingford: CABI, 275–290.

Morschhäuser J, 2010. Regulation of multidrug resistance in pathogenic fungi. *Fungal Genetics and Biology* **47**, 94–106.

Mullins JGL, Parker JE, Cools HJ *et al.*, 2011. Molecular modelling of the emergence of azole resistance in *Mycosphaerella graminicola* (MF Tuite, Ed,). *PLoS ONE* **6**, e20973.

Nei M, 1973. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences* **70**, 3321–3323.

Nei M, 1987. *Molecular evolutionary genetics*. New York, USA: Columbia Univ. Press.

News Room, 2019. Researchers confirm fungal resistance to strobilurins. *eFarmsNew*.

ODEPA - Oficina de Estudios y Políticas Agrarias, 2019. Estadísticas productivas - Chile. *Estadísticas de cultivos agrícolas*.

Olivet JJ, Villalba J, Volpi J, 2017. Evaluation of spray nozzles for fungicidal control of tan spot in wheat. *Crop and Pasture Science* **68**, 591.

Omrane S, Audéon C, Ignace A *et al.*, 2017. Plasticity of the MFS1 promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici* (AP Mitchell, Ed,). *mSphere* **2**.

Omrane S, Sghyer H, Audéon C *et al.*, 2015. Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates. *Environmental Microbiology* **17**, 2805–2823.

OPYPA - Oficina de Programación y Políticas Agropecuarias, 2019. Anuario Estadístico Agropecuario 2019 - Uruguay. *Estadísticas agropecuarias*.

Owen WJ, Yao C, Myung K *et al.*, 2017. Biological characterization of fenpicoxamid, a new fungicide with utility in cereals and other crops. *Pest Management Science* **73**, 2005–2016.

Parker SR, Welham S, Paveley ND, Foulkes J, Scott RK, 2004. Tolerance of Septoria leaf blotch in winter wheat. *Plant Pathology* **53**, 1–10.

Peakall R, Smouse PE, 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for

teaching and research--an update. *Bioinformatics* **28**, 2537–2539.

Pieczul K, Świerczyńska I, 2015. Fungicide sensitivity of *Mycosphaerella graminicola* (*Septoria tritici*) isolates. *Progress in Plant Protection* **55**, 316–320.

Ponomarenko A, Goodwin SB, Kema GHJ, 2011. Septoria tritici blotch (STB) of wheat. *Plant Health Instructor*, 1–7.

Posada D, 2008. jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* **25**, 1253–1256.

Pritchard JK, Stephens M, Donnelly P, 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–59.

Quaedvlieg W, Kema GHJ, Groenewald JZ *et al.*, 2011. *Zymoseptoria* gen. nov.: a new genus to accommodate *Septoria* like species occurring on graminicolous hosts. *Persoonia - Molecular Phylogeny and Evolution of Fungi* **26**, 57–69.

Rehfus A, Strobel D, Bryson R, Stammler G, 2018. Mutations in sdh genes in field isolates of *Zymoseptoria tritici* and impact on the sensitivity to various succinate dehydrogenase inhibitors. *Plant Pathology* **67**, 175–180.

Roohparvar R, Mehrabi R, Van Nistelrooy JG, Zwiers L-H, De Waard MA, 2008. The drug transporter MgMfs1 can modulate sensitivity of field strains of the fungal wheat pathogen *Mycosphaerella graminicola* to the strobilurin fungicide trifloxystrobin. *Pest Management Science* **64**, 685–693.

Roohparvar R, De Waard MA, Kema GHJ, Zwiers L-H, 2007. MgMfs1, a major facilitator superfamily transporter from the fungal wheat pathogen *Mycosphaerella graminicola*, is a strong protectant against natural toxic compounds and fungicides. *Fungal Genetics and Biology* **44**, 378–388.

Rosa M, Constanza M, Schalamuk S, 2013. Recent advances on integrated foliar disease management with special emphasis in Argentina wheat production. In: Nita M, ed. *Fungicides - Showcases of Integrated Plant Disease Management from Around the World*. InTech, 13.

Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC *et al.*, 2017. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution* **34**, 3299–3302.

Russell PE, 2004. *Sensitivity baselines in fungicide resistance research and management*. Brussels, Belgium: FRAC - Fungicide Resistance Action Committee.

Saintenac C, Lee W-S, Cambon F *et al.*, 2018. Wheat receptor-kinase-like protein Stb6 controls gene-for-gene resistance to fungal pathogen *Zymoseptoria tritici*. *Nature Genetics* **50**, 368–374.

Sánchez-Vallet A, McDonald MC, Solomon PS, McDonald BA, 2015. Is *Zymoseptoria tritici* a hemibiotroph? *Fungal genetics and biology : FG & B* **79**, 29–32.

Sanderson FR, 1972. A Mycosphaerella species as the ascogenous state of *Septoria tritici* Rob. and Desm. *New Zealand Journal of Botany* **10**, 707–709.

Schewe T, Lyr H, 1995. Mechanism of action of carboxin fungicides and related compounds. In: Lyr H, ed. *Modern Selective Fungicides*. New York: Gustav Fischer, 595.

Scholz R, Pereyra S, Garcia R, Germán S, 2019. Identificación de razas de roya de la hoja del trigo presentes en Uruguay durante 2011-2012. *Agrociencia Uruguay* **23**, 9.

Schuh W, 1990. Influence of tillage systems on disease intensity and spatial pattern of Septoria leaf blotch. *Phytopathology* **80**, 1337.

Scott PR, Sanderson FR, Benedikz PW, 1988. Occurrence of *Mycosphaerella graminicola*, teleomorph of *Septoria tritici*, on wheat debris in the UK. *Plant Pathology* **37**, 285–290.

Shaw MW, 1987. Assessment of upward movement of rain splash using a fluorescent tracer method and its application to the epidemiology of cereal pathogens. *Plant Pathology* **36**, 201–213.

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**, 882–899.

Shetty NP, Kristensen BK, Newman M-A, Møller K, Gregersen PL, Jørgensen HJL, 2003. Association of hydrogen peroxide with restriction of *Septoria tritici* in resistant wheat. *Physiological and Molecular Plant Pathology* **62**, 333–346.

Shiferaw B, Smale M, Braun H-J, Duveiller E, Reynolds M, Muricho G, 2013. Crops that feed the world. Past successes and future challenges to the role played by wheat in global food security. *Food Security* **5**, 291–317.

Siah A, Bomble M, Tisserant B *et al.*, 2018. Genetic structure of *Zymoseptoria tritici* in Northern France at region, field, plant, and leaf layer scales. *Phytopathology* **108**, 1114–1123.

Sierotzki H, 2015. Respiration Inhibitors: Complex III. In: *Fungicide Resistance in Plant Pathogens*. Tokyo: Springer Japan, 119–143.

Simón MR, Cordo C a., Castillo NS, Struik PC, Börner A, 2012. Population structure of *Mycosphaerella*

graminicola and location of genes for resistance to the pathogen: Recent advances in Argentina. *International Journal of Agronomy* **2012**, 1–7.

Simón MR, Perelló AE, Cordo CA, Struik PC, 2002. Influence on yield, yield components, and test weight of wheat under two nitrogen fertilization conditions. *Crop Science* **42**, 1974.

Simpson EH, 1949. Measurement of Diversity. *Nature* **163**, 688–688.

Stammler G, Brix HD, Nave B, Gold R, Schoefl U, 2007. Studies on the biological performance of Boscalid and its mode of action. In: Dehne HW, Deising HB, Gisi U, Kuck KH, Russel PE, Lyr LH, eds. *Modern Fungicides and Antifungal Compounds V - 15th International Reinhardsbrunn Symposium*. Friedrichroda, Germany: Spectrum Phytomedizin, 360.

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**, 1448–1456.

Stammler G, Wolf A, Glaettli A, Klappach K, 2015. Respiration Inhibitors: Complex II. In: Ishii H, Hollomon DW, eds. *Fungicide Resistance in Plant Pathogens*. Tokyo: Springer Japan, 105–117.

Steinberg G, 2015. Cell biology of *Zymoseptoria tritici*: Pathogen cell organization and wheat infection. *Fungal Genetics and Biology* **79**, 17–23.

Steinhauer D, Salat M, Frey R *et al.*, 2019. A dispensable paralog of succinate dehydrogenase subunit C mediates standing resistance towards a subclass of SDHI fungicides in *Zymoseptoria tritici* (Y-H Lee, Ed,). *PLOS Pathogens* **15**, e1007780.

Stergiopoulos I, van Nistelrooy JG, Kema GH, De Waard MA, 2003. Multiple mechanisms account for variation in baseline sensitivity to azole fungicides in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* **59**, 1333–1343.

Stewart TM, Perry AJ, Evans MJ, 2014. Resistance of *Zymoseptoria tritici* to azoxystrobin and epoxiconazole in the lower North Island of New Zealand. *New Zealand Plant Protection* **67**, 304–313.

Stocco MC, 2014. Control biológico de *Mycosphaerella graminicola*, patógeno del trigo, con cepas de *Trichoderma harzianum* caracterizadas por su morfología, fisiología, actividad enzimática y molecular. Universidad Nacional de La Plata.

Stoddart JA, Taylor JF, 1988. Genotypic diversity: estimation and prediction in samples. *Genetics* **118**, 705–11.

Stukenbrock EH, Banke S, Javan-Nikkhah M, McDonald BA, 2006. Origin and domestication of the fungal wheat pathogen *Mycosphaerella graminicola* via sympatric speciation. *Molecular Biology and Evolution* **24**, 398–411.

Suffert F, Sache I, Lannou C, 2011. Early stages of Septoria tritici blotch epidemics of winter wheat: build-up, overseasoning, and release of primary inoculum. *Plant Pathology* **60**, 166–177.

Sundqvist L, Keenan K, Zackrisson M, Prodöhl P, Kleinhans D, 2016. Directional genetic differentiation and relative migration. *Ecology and Evolution* **6**, 3461–3475.

Sykes EM, Sackett KE, Severns PM, Mundt CC, 2018. Sensitivity variation and cross-resistance of *Zymoseptoria tritici* to azole fungicides in North America. *European Journal of Plant Pathology* **151**, 269–274.

Tajima F, 1983. Evolutionary relationship of DNA sequences in finite populations. *Genetics* **105**, 437–60.

van Toor R, Viljanen-Rollinson S, Rahman A, Teulon D, 2013. Agrichemical use on wheat and barley crops in New Zealand in 2008–09. *New Zealand Journal of Crop and Horticultural Science* **41**, 9–22.

Torriani SFF, Brunner PC, McDonald BA, Sierotzki H, 2009. QoI resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*. *Pest Management Science* **65**, 155–162.

Tvaružek L, Horáková P, Ji L, 2012. Resistance behaviour of *Septoria tritici* to some fungicides in the territory of the Czech Republic. *Acta Agrobotanica* **58**, 79–84.

de Waard MA, Andrade AC, Hayashi K, Schoonbeek H, Stergiopoulos I, Zwiers L-H, 2006. Impact of fungal drug transporters on fungicide sensitivity, multidrug resistance and virulence. *Pest Management Science* **62**, 195–207.

White GA, Georgopoulos SG, 1992. Target sites of carboxamides. In: Koller W, ed. *Target sites of fungicide action*. London: CRC Press, 328.

Wiese M V., 1987. *Compendium of Wheat Diseases*. St. Paul, Minessota: The American Phytopathological Society.

Wright S, 1978. Variability within and among natural populations. In: *Evolution and the genetics of populations*. University of Chicago Press, 580.

Yamaguchi I, Fujimura M, 2005. Recent topics on action mechanisms of fungicides. *Journal of Pesticide*

Science **30**, 67–74.

Yamashita M, Fraaije B, 2018. Non-target site SDHI resistance is present as standing genetic variation in field populations of *Zymoseptoria tritici*. *Pest Management Science* **74**, 672–681.

Young DH, 2015. Anti-tubulin Agents. In: Ishii H, Hollomon DW, eds. *Fungicide Resistance in Plant Pathogens*. Tokyo: Springer Japan, 93–103.

Young DH, Wang NX, Meyer ST, Avila-Adame C, 2018. Characterization of the mechanism of action of the fungicide fenpicoxamid and its metabolite UK-2A. *Pest Management Science* **74**, 489–498.

Zhan J, Kema GH., Waalwijk C, McDonald B., 2002. Distribution of mating type alleles in the wheat pathogen *Mycosphaerella graminicola* over spatial scales from lesions to continents. *Fungal Genetics and Biology* **36**, 128–136.

Zhan J, McDonald B., 2004. The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola*. *Fungal Genetics and Biology* **41**, 590–599.

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**, 286–297.

Zhan J, Stefanato FL, MCDonald BA, 2006. Selection for increased cyproconazole tolerance in *Mycosphaerella graminicola* through local adaptation and in response to host resistance. *Molecular Plant Pathology* **7**, 259–268.

Zhong Z, Marcel TC, Hartmann FE *et al.*, 2017. A small secreted protein in *Zymoseptoria tritici* is responsible for avirulence on wheat cultivars carrying the Stb6 resistance gene. *New Phytologist* **214**, 619–631.

Ziogas BN, Malandrakis AA, 2015. Sterol biosynthesis inhibitors: C14 demethylation (DMIs). In: Ishii H, Hollomon DW, eds. *Fungicide Resistance in Plant Pathogens*. Tokyo: Springer Japan, 199–216.

Ziv O, Eyal Z, 1977. Assessment of yield component losses caused in plants of spring wheat cultivars by selected isolates of *Septoria tritici*. *Phytopathology* **68**, 791–796.

Zwiers LH, Stergiopoulos I, Gielkens MMC, Goodall SD, De Waard MA, 2003. ABC transporters of the wheat pathogen *Mycosphaerella graminicola* function as protectants against biotic and xenobiotic toxic compounds. *Molecular Genetics and Genomics* **269**, 499–507.