AN ABSTRACT OF THE DISSERTATION OF

Christina H. Hagerty for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on July 14, 2016.

Title: Dynamics of *Zymoseptoria tritici* Fungicide Resistance in the Willamette Valley of Oregon.

Abstract approved:

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Christopher C. Mundt

The development of resistance to multiple fungicide classes is currently limiting disease management options for many pathogens, while the discovery of new fungicide classes has become less frequent. To address this pressing issue, a three-part study with different layers of complexity, objectives, and experimental approaches was conducted using the model fungicide resistance pathosystem: *Zymoseptoria tritici*. The virulence of azoxystrobin-resistant *Zymoseptoria tritici* was tested in greenhouse assays, and results indicated that azoxystrobin-resistant *Z. tritici* populations were less virulent. Results of a series of field inoculations with fungicide-resistant, fungicide-sensitive, and resistant/sensitive mixtures indicated that the competitive ability and fitness of azoxystrobin-resistant *Z. tritici in vivo* was influenced by environmental factors. A large-scale hierarchical survey of commercial wheat fields in the Willamette Valley of Oregon was executed to monitor seasonal changes and spatial variation of propiconazole- and azoxystrobin- resistant *Z. tritici*.

An increase of azoxystrobin resistance was recorded over the two-year study. In contrast, propiconazole resistance stayed relatively stable over time. Both fungicide-resistant phenotypes were found to be spatially random, and the greatest amount of spatial variation occurred at the smallest hierarchical scale of the survey. Results of this research represent a broad effort to understand fitness trade-offs associated with mutations, as well as a more focused effort to understand local dynamics of *Z. tritici* fungicide resistance in the in the Willamette Valley of Oregon.

Dynamics of *Zymoseptoria tritici* Fungicide Resistance in the Willamette Valley of Oregon

by
Christina H. Hagerty

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APPROVED:

Major Professor, representing Botany and Plant Pathology

Head of the Department of Botany and Plant Pathology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Christina H. Hagerty, Author

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This dissertation is dedicated to my late grandmother Dorothy Dean Hagerty, who went back to school at age 57 to obtain her PhD.

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CHAPTER 1: INTRODUCTION

Christina H. Hagerty

PREFACE

Fungicide resistance is defined as a stable heritable trait that results in insensitivity of a fungus to a fungicide (McGrath 2004). The build-up of fungicide resistance in an agricultural landscape can cause pathogen control failure, which can ultimately lead to crop loss. Fungicide resistance arises from random mutations in a population and can rapidly increase in frequency due to the strong selection pressure exerted by the fungicide. Central ecological and evolutionary theory dictates that mutations for resistance come at a fitness trade off in the absence of selection pressure.

Fitness of the resistant individual is important because it determines persistence and competitive ability over time. Which in turn, is relevant for understanding how resistance mutations might change population diversity and evolutionary biology of an organism. Furthermore, fitness is relevant for understanding the persistence of a resistant phenotype in an agricultural landscape.

I conducted a three-part study with different layers of complexity, objectives, and experimental approach. Chapter two details the simplest experiment of this study; we measured the virulence of azoxystrobin-resistant *Zymoseptoria tritici* in *in planta* greenhouse assays. We found reduced virulence of azoxystrobin-resistant *Z. tritici* populations in greenhouse assays and this study was published in Phytopathology.

Next, in chapter three, I added complexity to my research approach by conducting a series of large-scale field inoculations with fungicide -resistant, fungicide-sensitive, and resistant/sensitive mixtures. We found the competitive ability and fitness of azoxystrobin-resistant *Z. tritici in vivo* is influenced by environmental factors. Last, in Chapter four, we conducted a large-scale hierarchical survey of commercial wheat fields in the Willamette Valley of Oregon to monitor seasonal changes and spatial variation of propiconazole and azoxystrobin-resistant *Z. tritici*. We recorded an increase of azoxystrobin resistance over the two-year study. In contrast, propiconazole resistance stayed relatively stable over time. We also found that the fungicide resistance phenotype was spatially random organized, not aggregated within the commercial fields sampled, and the greatest amount of spatial variation occurred at the smallest hierarchical scale of the survey. This study is in review at Phytopathology.

When considered all together, the three chapters result in a complex picture relative to virulence, fitness, and competitive ability of fungicide-resistant *Z. tritici*. We hope results of this work, which used a model organism for evolutionary ecology, will help to elucidate biological patterns associated with resistance mutations in other systems. In addition, we hope results will contribute to the overall effort for fungicide resistance management in an agricultural landscape and provide a clear demonstration of how quickly a chemical can be lost to resistance.

ZYMOSEPTORIA TRITICI DISEASE CYCLE AND EVOLUTIONARY BIOLOGY

Zymoseptoria tritici (Desmazieres, 1842) is the causal agent of Septoria tritici blotch (STB) of bread wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum*

[L.] ssp. *Durum)*. *Z. tritici* is a heterothallic foliar ascomycete with a biotrophic phase early in the infection period, and a necrotrophic phase later in the infection period (Shetty et al. 2003). *Z. tritici* is ubiquitous in the Willamette Valley, as the Mediterranean climate favors annual epidemics that range from moderate to severe (Mundt et al. 1999).

Most features of the *Z. tritici* lifecycle favor the development of fungicide resistance. These features include a large effective population size, a sexual recombination phase, an asexual repeating cycle, and the long-distance dispersal of ascospores (McDonald and Mundt 2016). A dedicated effort to study and monitor *Z. tritrici* fungicide resistance dynamics in the Willamette Valley was needed. To complement *Z. tritici* monitoring efforts, a better understanding of the fitness and virulence trade-offs associated with fungicide-resistant *Z. tritici* is needed to understand the longevity and competitive ability of fungicide-resistant isolates within different spatial sales in an agricultural landscape.

Z. tritici epidemics in the Willamette Valley begin with a heterothallic mating system, or a "sexual stage". Ascocarps, the sexual fruiting body containing ascospores, are abundant on wheat chaff following summer harvest (DiLeone et al. 1997; Mundt et al. 1999). The peak ascospore infection period typically follows heavy autumn rains and lasts until early spring (Brown, Kellock, and Paddick 1978; DiLeone et al. 1997; Mundt et al. 1999). In the Willamette Valley, ascospore showers appear to peak in November (DiLeone et al. 1997). In eastern England, with a maritime climate similar to the Willamette Valley, ascospore showers peak in December and January (Scott, Sanderson, and Benedikz 1988). The abundance of

sexually produced spores early in the growing season provide a great deal of genetic diversity at the time of initial infection (Banke and Mcdonald 2005)(Banke and Mcdonald 2005)(Banke and Mcdonald 2005)(Banke and Mcdonald 2005), and this vast pool of genetic diversity is the foundation of evolutionary potential, increasing the risk of developing fungicide resistance.

In early January, at the third leaf stage, susceptible wheat cultivars can be found to have multiple pycnidia on every plant sampled. *Z. tritici* has a very large effective population size (Mundt et al. 1999). In a study by (Linde, Zhan, and McDonald 2002), lesions often contained from 2-8 different genotypes, and four out of five lesions sampled contained both mating types. Furthermore, it is estimated that a single field population size can range from 3,400 to 700,000 unique individuals (Zhan, Mundt, and McDonald 2001).

Sexual, wind-dispersed ascospores released from pseudothecia serve as the primary inoculum source of STB (Shaw and Royle 1989) (Figure 1.1). It is estimated that each pseudothecium can produce up to 200 ascospores. When ascospores land on susceptible host tissue in favorable humidity and temperature, germination occurs, and hyphal growth extends into the mesophyll tissues (Ponomarenko, Goodwin, and Kema 2011). The asexual latent period, depends on temperature, leaf wetness, and precipitation but generally is 20 ± 4 days depending on environmental conditions (Henze et al. 2007).

Z. tritici is capable of penetrating the wheat leaf directly through the cell walls of the epidermis, or more commonly through the stomata (Eyal 1987). *Z. tritici* thrives on nutrition obtained from the plant apoplast, then makes a rapid switch to

necrotrophic growth, which can be visualized with the rapid development of necrotic lesions. Within the necrotic lesion, asexual pycnidia develop above sub-stomatal spaces and can be seen with the naked eye as 0.5-2mm black dots organized in rows on the leaf surface (Eyal 1987; Ponomarenko, Goodwin, and Kema 2011). When humidity is high, pycnidia swell and exude cirrhi, a milky protein-rich substance containing conidia, which is splash dispersed within the wheat canopy to new infection sites. This portion of the disease cycle in the Willamette Valley can be observed when STB infections begin on the lower leaves of the canopy at primary and secondary leaf stage, and end on the flag leaf in severe epidemics through splash dispersal. *Z. tritici* epidemics are long: typically beginning in November and ending in June, as rainfall dwindles. The generation time of the STB asexual cycle is dependent upon growing degree days and rainfall (Henze et al. 2007). When conditions permit, 10+ asexual STB generations can occur in the Willamette Valley

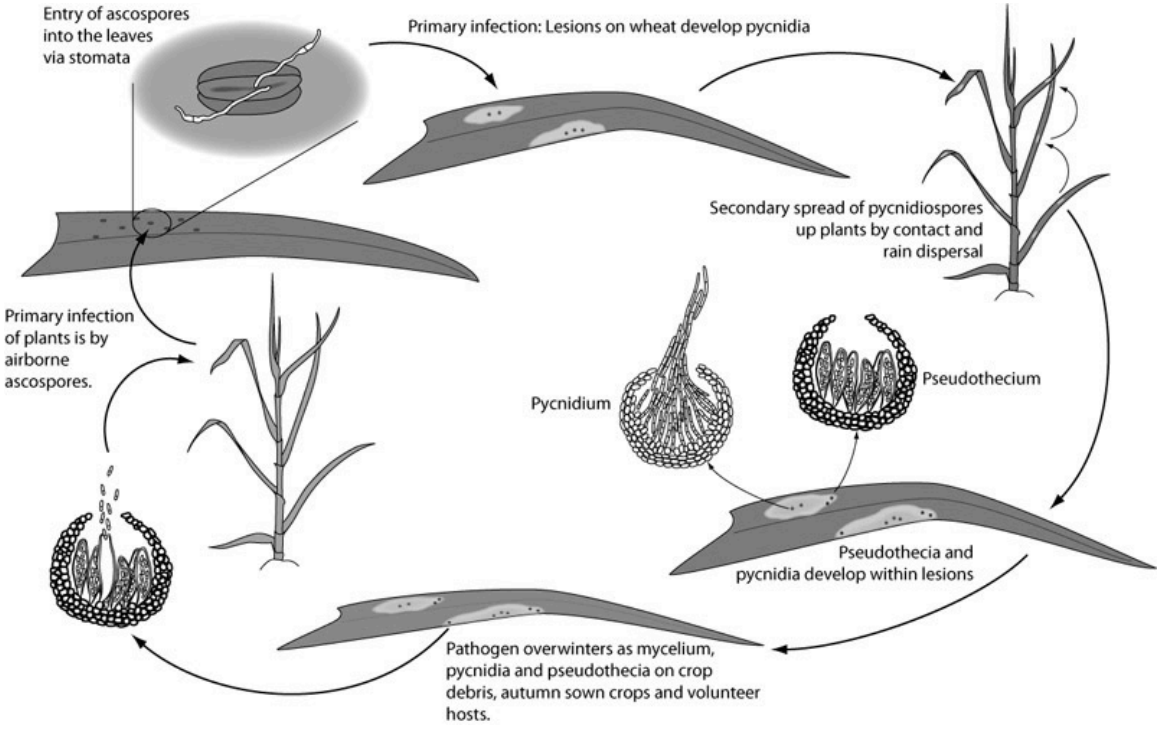

Figure 1.1. Illustrated disease cycle of Septoria tritici blotch caused by *Zymoseptoria tritici* (Ponomarenko, Goodwin, and Kema 2011 - permission requested).

HISTORY OF SEPTORIA TRITICI BLOTCH (STB) CONTROL IN THE WILLAMETTE VALLEY 1959-2016

Using the Pacific Northwest (PNW) Plant Disease Management Handbook archives located in the Oregon State University Plant Disease Clinic Library and other resources, a history of STB control in the Willamette Valley was assembled. In 1959, the first edition of the PNW handbook, it is reported that STB seldom causes serious losses in Oregon. With the relatively low importance of STB at that time, "crop rotation, summer fallow, and plowing under volunteer wheat to reduce the amount of disease" was recommended for disease control. This was true for STB of wheat worldwide where, prior to 1960, only occasional yield losses with economic impact were reported (Eyal 1987). The increase of STB economic damage after the 1960s is likely due to the adoption of new semi-dwarf, early maturing cultivars that were more susceptible to STB (Eyal 1987). New varieties with adequate STB resistance compared to the early "Green Revolution" varieties are released by modern wheat breeding programs regularly. However, the same features that make STB high risk for overcoming fungicides, also apply to host resistance, where ubiquitous presence of STB in the Willamette Valley and large population genetic diversity, make STB able to overcome resistance genes quickly. *Z. tritici* has overcome the resistance in all major winter wheat cultivars grown in the Willamette Valley over the last 20 years, regardless of whether the resistance was qualitative or quantitative (McDonald and Mundt 2016).

In 1975, the statement that STB is “not a threatening disease” was removed from the PNW handbook, and cultural controls were still recommended. In 1982 mancozeb was introduced in the PNW handbook as the first chemical control recommended for the control of STB. Mancozeb is a non-systemic multi-site protectant fungicide (Gullino et al. 2010). In the Willamette Valley, mancozeb did not improve yields, but did give improved test weights (Koepsell et al. 1983). In 1985 the first tank mix for the control of STB was recommended, as a mixture of mancozebmixed with benzimidizole (Benlate). Benzimidizole is a systemic fungicide that blocks microtubules in fungi (Salahuddin, Shaharyar, and Mazumder 2012). In 1988, a single application of propiconazole (Tilt) was introduced for the control of STB. Propiconazole is in the triazole family, and works as a demethylation inhibiting fungicide (DMI), which interferes with sterol biosynthesis in the fungus. In 1997, copper hydroxide (Kocide 3000), a multisite protectant, was recommended for septoria control for one year. In 1998, triadimefon (Bayleton) (DMI) was recommended. In 2000, azoxystrobin (Quadris), a quinone outside inhibitor (QoI) was recommended for STB control. In 2004, an azoxystrobin and propiconazole tank mix was recommended. The following year in 2005, a product blending azoxystrobin and propiconazole was released in a pre-mixed product (Quilt). In 2014, the succinate dehydrogenase inhibitor (SDHI) class became available for STB control. SDHIs were released first as Priaxor, a two-way mix with fluxapyroxad (SDHI) and pyraclostrobin (QoI). Shortly thereafter, Trivapro was released as a three-way mix with benzovindiflupyr (SDHI), azoxystrobin (QoI), and propiconazole (DMI); and Vertisan with the active ingredient penthiopyrad (SDHI).

In the Introduction sections of Chapters 2 and 4, literature regarding the general issues of fungicide resistance in plant pathology, experience with fungicide resistance to *Z. tritici* in Europe, and the more recent development of *Z. tritici* fungicide resistance in the Willamette Valley of Oregon is reviewed. The Introduction of Chapter 3 literature associated with the issue of measuring fitness costs in pathogens and other organisms is discussed.

CHAPTER 2: REDUCED VIRULENCE OF AZOXYSTROBIN-RESISTANT *ZYMOSEPTORIA TRITICI POPULATIONS IN GREENHOUSE ASSAYS*

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ABSTRACT

The development of resistance to multiple fungicide classes is currently limiting disease management options for many pathogens, while the discovery of new fungicide classes may become less frequent. In light of this, more research is needed to quantify virulence trade-offs of fungicide resistance in order to more fully understand the implications of fungicide resistance on pathogen fitness. The purpose of this study was to measure the virulence of azoxystrobin-resistant and -sensitive *Zymoseptoria tritici* populations collected from North and South Willamette Valley, Oregon, in 2012 and 2015. Inoculum mixtures of known fungicide-resistant phenotypes were used to simulate natural field conditions, where multiple genotypes exist and interact in close proximity. Six greenhouse inoculations were conducted over two years, and virulence of the isolate mixtures was evaluated *in planta*. We considered virulence to be "the degree of pathology caused by the organism" and visually estimated the percent area of leaf necrosis as a measure of virulence. In greenhouse conditions, a consistent association of reduced virulence with azoxystrobin-resistant *Z. tritici* isolate mixtures was observed. North Willamette Valley and South Willamette Valley populations did not differ in virulence.

INTRODUCTION

Chemical control has a significant role in helping farmers meet the increased demand for agricultural resources (Cooper and Dobson 2007), and fungicide use is increasing for many crops (Russell 2005). World sales of agricultural fungicides totaled 9.91 billion US dollars (USD) in 2010 (Hirooka and Ishii 2013), with 23% of

product sold for use on cereals (Phillips McDougall 2011). However, the development of resistance to multiple fungicide classes is currently limiting disease management options for many pathogens (Knight et al. 1997; Leadbeater 2015), while the discovery of new fungicide classes may become less frequent (Cools and Fraaije 2008; Leadbeater 2015; Russell 2005; Sierotzki, Wullschleger, and Gisi 2000; Stammler et al. 2008). Furthermore, it takes an estimated 250 million USD and nine years of research and development to bring a new active ingredient to market (Phillips McDougall 2013). In absence of new fungicide classes or improved strategies to manage resistance, a rapid shift to novel disease control would be required to maintain global food security.

Antimicrobial resistance as a biological trade-off, with resistant genotypes less fit than sensitive genotypes in the absence of fungicide selection , remains a central theory of medical and agricultural pathology (Andersson and Levin 1999; Gagneux et al. 2006; Vila-Aiub, Neve, and Powles 2009). Several resistance management techniques have been proposed to reduce selection for fungicide-resistant genotypes, and the effectiveness of these approaches will be influenced by trade-offs (Mikaberidze and McDonald 2015; Shaw 2006; Van Den Bosch et al. 2014). The invasion and persistence of fungicide-resistant genotypes in a landscape depends directly on the relative fitness of the resistant genotype compared to the wild type (Gubbins and Gilligan 1999; Zhan and McDonald 2013), as measured by the selection coefficient (Van Den Bosch et al. 2014). Some models assume that no fitness costs are associated with fungicide resistance (Levy, Levi, and Cohen 1983; Milgroom and Fry 1988; Shaw 1989, 1993), but the development of more accurate

trial-based fitness and virulence estimates could be a positive addition to improve mathematical model predictions. More work is needed in this specific area, as fitness costs may have to be quite large to affect the rate of buildup of fungicide resistance (Shaw 2006).

Zymoseptoria tritici causes Septoria tritici blotch (STB), a devastating disease of wheat worldwide, with yield losses up to 40% in severe epidemics (Eyal 1987). STB is currently the most important wheat disease in the European Union (Fones and Gurr 2015), the largest producer of wheat globally (Statistica 2015). STB is also described as a yield-limiting factor in the Willamette Valley of Oregon (DiLeone et al. 1997), where the isolates used in this study were obtained. Effective fungicides are a crucial tool farmers use to control *Z. tritici* epidemics at reasonable levels. However, the emergence of fungicide resistance in *Z. tritici* to the strobilurin fungicide class is threatening the current practices for STB control in many major wheat producing regions (Drabešová et al. 2013; Estep et al. 2013; Siah et al. 2014; Torriani et al. 2009). In light of this, we need to know more about virulence trade-offs of fungicide resistance in order to more fully understand the implications of fungicide resistance to a pathogen's fitness.

A high level of resistance to azoxystrobin is conferred by the G143A mutation in the mitochondrial gene encoding cytochrome B, where a single base pair mutation at codon 143 substitutes glycine by alanine (Gisi et al. 2002). In *Z. tritici,* the G143A mutation is heritable and can arrise independently (Torriani et al. 2009). The G143A mutation leading to quinone outside inhibitor (QoI) resistance is also common in other plant pathogenic ascomycetes, including *Blumeria graminis*, *Magnaporthe*

grisea, and *Venturia inaequalis* (Grasso et al. 2006). Associations of the G143A mutation with pathogen fitness have been reported as positive in *Mycosphaerella graminicola* of wheat (Fraaije et al. 2005), neutral in *Magnaporthe grisea* of rice and *Erysiphe graminis* of cereals (Avila-Adame and Köller 2003; Chin et al. 2001), and negative in *Magnaporthe oryzae* of ryegrass (Ma and Uddin 2009) compared with wild-type strains.

Approximately 93% of the *Z. tritici* population in the Willamette Valley was resistant to azoxystrobin as of 2015 (Hagerty et. al. unpublished data) This is not surprising, given the biology and lifecycle of *Z. tritici* in the Willamette Valley (Mundt et al. 1999), the recent history of fungicide use in this region (Estep et al. 2015), and the status of QoI-resistant *Z. tritici* in other regions (Drabešová et al. 2013; Siah et al. 2014; Torriani et al. 2009). The risk for development of fungicide resistance of *Z. tritici* in the Willamette Valley is due to four main characteristics: sexual recombination leading to vast genetic diversity and thus evolutionary potential, large effective population size, ubiquitous presence of *Z. tritici* in the Willamette Valley, and long epidemics with many repeating asexual cycles (FRAC 2005; Ponomarenko, Goodwin, and Kema 2011). These characteristics, in concert, put *Z. tritici* at high risk for developing fungicide resistance (FRAC 2005).

The purpose of this study was to measure the virulence of azoxystrobin-resistant and -sensitive *Z. tritici* populations collected from different areas of the Willamette Valley, in different years. We consider virulence to be "the degree of pathology caused by the organism" (Andrivon 1995). Our findings indicate that azoxystrobin-

resistant *Z. tritici* isolate mixtures are more virulent under greenhouse conditions than wild-type *Z. tritici* isolate mixtures *in planta*.

MATERIALS AND METHODS

Isolate acquisition and selection. The isolates collected in 2012 originated from two source populations in the Willamette Valley of western Oregon, USA. The collections were genotyped in 2012 and were described by Estep et al. (2015). Flag leaves naturally infected by *Z. tritici* were sampled during 2012 from fungicide trials, approximately seven weeks after the final fungicide applications. Two trials in the Willamette Valley were sampled; one trial was within a commercial wheat field in North Willamette Valley, and the other trial was at the Hyslop Field Laboratory in South Willamette Valley. Collection sites were approximately 100 km apart. Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) protocols were used to screen isolates for the presence or absence of the G143A mutation for complete resistance to azoxystrobin (Estep et al. 2015). Using isolates collected and genotyped by Estep et al. (2015), 12 isolates with the G143A substitution and 12 isolates without the G143A substitution were selected from each of the source populations, North Valley and South Valley. The 12 isolates differing in the G143A substitution and source population (48 isolates total) were used to make four inoculum treatments in a factorial design (see Treatments below). These isolates were chosen randomly from each of the North and South populations, without regard to the treatments applied to the original plots of origin (some plots received azoxystrobin, while others did not).

The resistant phenotype was confirmed in Oregon by transferring each isolate onto two Petri plates – one plate with non-amended yeast malt agar (YMA) (Hardy Diagnostics, California, USA) and one plate with YMA amended with 1 μg/ml technical grade azoxystrobin (Syngenta, Basel, Switzerland). Three days after plating, isolate growth on the azoxystrobin-amended plate was compared to that of the non-amended plate. If the azoxystrobin-amended plate had reduced growth visually compared to the non-amended plate, the isolate was scored as sensitive. If the amended plate had the same growth visually compared to the non-amended plate, the isolate was scored as resistant. Phenotypic results matched genotypic results of Estep et al. (2015) at 100% accuracy.

A second group of isolates was collected in 2015 and originated from six commercial fields in the Willamette Valley: three fields from North Willamette Valley, and three fields from South Willamette Valley. There was an average of approximately 150 km between the North and South fields, and an average of approximately 45 km between fields within each of these two regions. To collect the isolates, workers walked two, 300-m transects and gathered flag leaves naturally infected by *Z. tritici* at every 30-m interval. Leaves were collected approximately three weeks after the final fungicide applications in the fields. The collections were phenotyped for resistance to axoystrobin in 2015 as described above.

Using isolates collected and phenotyped in vitro, 10 isolates resistant to azoxystrobin and 10 isolates sensitive to azoxystrobin were selected from each of the source locations, North Valley and South Valley, totaling 40 isolates. The 10 isolates differing in azoxystrobin resistance and source population were used to make four

inoculum treatments in a factorial design (see Treatments below). Ten isolates were used in year 2, rather than 12, because the proportion of axoxystrobin resistance had become so high by 2015 that collections were limited by availability of susceptible isolates.

Treatments included isolates collected in 2012 and 2015 to gain insight into differences that may be the result of additional evolutionary processes, such as the build- up of compensatory mutations in fungicide-resistant isolates (Jeger, Wijngaarden, and Hoekstra 2008).

Greenhouse trials. Pathogen populations were evaluated during winter 2014-2015, and autumn 2015; for clarity and brevity, these will be referred to as "year 1" trials and "year 2" trials, respectively. Isolates collected in 2012 were tested six times (two trials in year 1, and four trials in year 2) to assess repeatability of the methodology. Isolates collected in 2015 were tested twice (two trials in year 2).

Treatments. In year 1, inoculum treatments were a two-by-two factorial of isolates collected in 2012 differing in azoxystrobin phenotype (resistant or sensitive) and location of origin (North Willamette Valley or South Willamette Valley). In year 2, four inoculations occurred, two of which used South Valley isolates, and two of which used North Valley isolates. The South Valley treatments were a factorial of two pathogen phenotypes (azoxystrobin-sensitive or resistant) times two years of collection from the South Willamette Valley (2012 or 2015). The North Valley treatments were identical to the South Valley treatments, except for location of initial collection.

There were six replicates in each greenhouse trial, with a pot of five seedlings being the experimental unit. Plants were raised in 10-cm plastic pots filled with potting soil (Metro-Mix 840, SunGro Horticulture, Vancouver, Canada). In year 1, potting soil was amended with Osmocote 18-6-12 extended time-release fertilizer (Scott-Sierra Horticultural Products Co., Marysville, OH) at a rate of approximately 1 g per pot at seeding. In year 2, potting soil was amended with Miracle-Gro liquid fertilizer (Scott-Sierra Horticultural Products Co., Marysville, OH) weekly starting two weeks after seeding at a rate of approximately 100ml/pot of the labeled dilution rate of 3.9 ml per L. The plants were grown under high-pressure sodium lighting to extend the day-length to 16 h. Pots were grown on cafeteria trays and bottom-watered daily. Approximately seven seeds of wheat cultivar 'Skiles' (Flowers et al. 2010) were sown in each pot and seedlings were thinned to five plants per plot at about 10 days after planting. At 18 days after seeding, the five seedlings per plot were supported with a wire cage to keep the leaves in an upright position. In year 2, to control for aphids, plants were treated when necessary with acephate systemic insecticide (Bonide Products Incorporated, Oriskany, NY).

Inoculum preparation and inoculation. Isolates of *Z. tritici* were cultured individually for four days on fresh yeast-malt agar (YMA). Spores of each isolate were rinsed from YMA dishes with sterile deionized water and mixed by treatment. Isolate mixtures were adjusted to one 75 ml portion with a concentration of 10^6 spores per ml. Each 75 ml portion of inoculum received one drop of Tween 20 surfactant to help the inoculum adhere to the leaf surface. When plants were 21 days old, each spore treatment was applied to a group of six pots by means of spray bottle and

phonographic turntable (Dual Corporation, Germany). Seventy-five milliliters were applied until run-off to the six plants atop the turntable. After inoculation, pots were completely randomized and kept in a custom fabricated greenhouse bench-top mist chamber, utilizing home ultrasonic humidifiers (The Holms Group, Inc. Milford, MA) for 96h. Non-inoculated pots were treated the same as inoculated plots, in order to control for contamination in the experiments.

Greenhouse disease assessments. In year 1, plants were evaluated 21 days after inoculations. In year 2, plants were evaluated 23 days after inoculations. To evaluate each plant, the second leaf from the base of the plant was destructively sampled by physically removing the leaf from the plant. The detached leaf was then visually estimated for the percent area of leaf necrosis. All leaf measurements from a given experiment were taken on the same day.

STATISTICAL ANALYSIS

All analyses were performed in R (version 3.1.2, 2014-10-31) using the R studio graphical user interface (GUI) (Version 0.98.1091 RStudio, Inc.). The *lm* function in the *lme4* package was used to build a linear model and *anova* was then used to evaluate terms of the model. Separate analyses were conducted for each of the three main trials. Residual plots indicated no need for data transformation. For year 1 and for the year 2 South isolate trial, experiments were combined and experiment was included as a co-factor because there were no significant ($P<0.05$) interactions of experiment with main effects. For the year 2 North isolate trial, experiments were analyzed separately because there were significant ($P<0.05$) interactions of experiment with main effects.

RESULTS

In the trial including only isolate mixtures from 2012 (year 1), fungicide-sensitive inoculum was more virulent than fungicide-resistant inoculum ($P < 0.0001$). The source of the inoculum (North versus South) did not have an effect on virulence ($P = 0.5530$) (Table 2.1). There were no significant interactions among sources of variation.

In the trial including only South Valley experiments (year 2), fungicide-sensitive inoculum was more virulent than fungicide-resistant inoculum ($P = 0.0002$). Inoculum collected in 2012 was more virulent than inoculum collected in 2015 ($P=0.0002$) (Table 2.2). There were no significant interactions among sources of variation.

In the trial including only North Valley experiments (year 2), experiments were analyzed separately, owing to significant experiment × year and experiment × year × isolate resistance interactions (Table 2.4). Results from the two experiments were qualitatively similar; however, fungicide-sensitive inoculum was more virulent than fungicide-resistant inoculum in both experiments ($P<0.0001$ for experiment 1 and experiment 2). No difference between virulence and year collected was observed in experiment 1 ($P = 0.7472$); however, inoculum collected in 2012 was more virulent than inoculum collected in 2015 in experiment 2 ($P=0.0041$). The isolate resistance × year collected interaction was not significant in experiment 1 ($P= 0.2281$), but was significant in experiment 2 ($P=0.0038$) (Tables 2.2 and 2.3).

In all analyses, resistant inoculum was less virulent than sensitive inoculum (Figures 2.1-2.4).

DISCUSSISON

In greenhouse conditions, we observed a consistent association between reduced virulence and azoxystrobin resistance of *Z. tritici* isolates. In all analyses, resistant inoculum was less virulent than sensitive inoculum, with the source location of inoculum collection not impactful on virulence and with inoculum collected in 2012 slightly more virulent than inoculum collected in 2015. The difference in virulence between fungicide-resistant and fungicide-sensitive isolates was large and repeatable, regardless of region (North Willamette Valley versus South Willamette Valley) or year (2012 versus 2015) of collection. We used mixtures of 10-12 isolates per treatment in each experiment to allow us to more closely simulate natural field conditions, where multiple genotypes exist and interact in close proximity (Linde, Zhan, and McDonald 2002). Utilizing mixtures also allowed us to test a larger total number of isolates with the same resources.

The plant fertilization scheme was changed in the second year of the study, resulting in visibly healthier and more uniform plants. This improvement in procedures likely lowered overall disease severity and increased time to optimum disease expression in year 2, given that *Z. tritici* has a significant necrotrophic stage (Ponomarenko, Goodwin, and Kema 2011). These differences can be visualized in box plots between the two years (Figures 2.1-2.3). In addition, mean square errors in the ANOVAs are about half or less in the second year as compared with the first year (Tables 2.1-2.3). Nonetheless, the effect of azoxystrobin resistance on virulence did not change qualitatively between the two years of greenhouse studies.

Previous observations in both experimental field plots (Hayes et al. 2015) and commercial fields (Hagerty and Mundt 2015) suggest that azoxystrobin resistance may be associated with increased fitness in the field, which conflicts with results of our greenhouse studies reported here. This conflict has several possible explanations. First, though virulence is expected to be positively associated with high horizontal transmission (Lipsitch, Siller, and Nowak 1996; Stewart, Logsdon, and Kelley 2005) and fitness measures such as pycnidia density and percent leaf area covered by lesions have been found to be positively correlated for *Z. tritici* (Stewart and McDonald 2014), there may not be a complete correlation between virulence and reproductive fitness for this pathogen. Unfortunately, pycnidial production in the greenhouse, a more direct measure of fitness, was too inconsistent for accurate estimation in our study. Second, our studies measured resistant isolate mixtures and sensitive isolate mixtures separately, which does not account for potential competitive differences between resistant and sensitive inoculum. Future work includes the evaluation of mixtures of resistant and sensitive isolates. Finally, it is possible that isolates performed differently in a greenhouse environment than they would in the field.

We utilized isolates collected in both 2012 and 2015 to test the hypothesis of compensatory mutations (Andersson and Levin 1999; Brunner, Stefanato, and Mcdonald 2008) to ameliorate virulence reductions of resistant phenotypes that were apparent in 2012. The hypothesis of compensatory mutations was rejected, as similar patterns in virulence between azoxystrobin-resistant and azoxtrobin-sensitive isolates were observed from the 2012 and 2015 collections. Isolates collected in 2015 had

gone through three additional years of evolutionary forces, though it is possible that amelioration may yet develop over time. Averaged over fungicide phenotypes, results from year 2 South trials suggest that isolates collected in 2015 may be less virulent than isolates collected in 2012. These results however are not consistent with year 2 North trials. This result could be due to either differing selective pressures or to sampling effects between the two years.

The G143a mutation occurs in the mitochondrial genome (Torriani et al. 2009), which is maternally inherited and does not undergo allelic recombination. Thus, the virulence of the mitochondrial background(s) in which the G143A mutation occurred could be the primary determinant of the virulence reduction that we measured, rather than the G143A mutation *per se*. Haplotype diversity of *Z. tritici* in Oregon isolates containing the G143A mutation are dramatically less diverse than wild type isolates (Estep et al. 2015). A strong association between azoxystrobin resistance and virulence may not have been detected had the mutation been present in the nuclear genome of *Z. tritici*.

The fitness cost of fungicide resistance plays an important role in resistance management tactics (Mikaberidze and McDonald 2015; Shaw 2006; Van Den Bosch et al. 2014). In our study, azoxystrobin-resistant isolates had lower virulence compared to azoxystrobin-sensitive isolates in the greenhouse, when using percent leaf necrosis as a measure of virulence. As noted earlier, associations between fitness and azoxystrobin resistance have been highly variable (Avila-Adame and Köller 2003; Chin et al. 2001; Fraaije et al. 2005; Ma and Uddin 2009). In the field of herbicide resistance, the effect and depth of the fitness penalty of resistance depends

on many factors, including the environment, the genetic background in which the mutation occurs, the specific mutation conferring resistance, and the resistance mutation itself (Bergelson and Purrington 1996; Délye et al. 2013; Menchari et al. 2008; Vila-Aiub, Neve, and Powles 2009). The same is almost certainly to be true for fungicide resistance. Furthermore, conclusions about fitness and virulence trade-offs can depend on the trait measured to quantify pathogenicity and fecundity.

In summary, we have found a strong and highly consistent association between azoxystrobin resistance and reduced virulence in *Z. tritici* in the greenhouse. Work remains to be done to determine the relevance of these results to competition among resistant and sensitive strains in the field, a factor crucially important to improve resistance management strategies and maintain an important disease management resource.

ACKNOWLEDEMENTS

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Table 2.1. Analysis of variance for virulence of *Zymoseptoria tritici* isolate mixtures (12 isolates each) collected in the Willamette Valley of Oregon in 2012, Each of two experiments consisted of a factorial two inoculum mixture sources (North and South Valley) and two inoculum mixture phenotypes (azoxystrobin-resistant and sensitive) evaluated in six replicates in the greenhouse.

Source of variation	df	Mean Sq	*F* value	*P* value
Experiment	1	0.2080	15.64	0.0004
Inoculum mixture source	1	0.0049	0.37	0.5495
Inoculum mixture phenotype	1	0.6396	48.09	0.0000
Experiment:inoculum mixture source	1	0.0519	3.90	0.0574
Experiment:inoculum mixture phenotype	1	0.0008	0.06	0.8058
Inoculum mixture source:inoculum mixture phenotype	1	0.0053	0.39	0.5345
Experiment:inoculum mixture source:inoculum mixture phenotype	1	0.0046	0.34	0.5631
Residuals	30	0.0133		

Table 2.2. Analysis of variance for virulence of *Zymoseptoria tritici* isolate mixtures (10 isolates each) collected from the South Willamette Valley, Oregon, differing in resistance to azoxystrobin and year of collection, each of two experiments consisted of a factorial of two years of collection (2012 and 2015) and two inoculum mixture phenotypes (azoxystrobin-resistant and sensitive) evaluated in six replicates in the greenhouse.

Source of variation	df	Mean Sq	*F* value	*P* value
Experiment	1	0.0899	16.91	0.0002
Year collected	1	0.0927	17.43	0.0002
Inoculum mixture phenotype	1	0.1267	23.83	0.0000
Experiment:year collected	1	0.0000	0.00	0.9800
Experiment:inoculum mixture phenotype	1	0.0000	0.00	0.9672
Year collelcted:inoculum mixture phenotype	1	0.0001	0.02	0.8887
Experiment:year collected:inoculum mixture phenotype	1	0.0110	2.07	0.1585
Residuals	39	0.0053		

Table 2.3. Analysis of variance for virulence of *Zymoseptoria tritici* isolate mixtures (10 isolates each) collected from the North Willamette Valley, Oregon and evaluated in two greenhouse experiments, each experiment was a factorial of two years of collection (2012 and 2015) and two inoculum mixture phenotypes (azoxystrobin-resistant and sensitive) evaluated in six replicates.

	Experiment 1				Experiment 2			
Source of variation	df	Mean Sq	*F* value	*P* value	df	Mean Sq	*F* value	*P* value
Inoculum mixture phenotype	1	0.2489	33.37	0.0000	1	0.3064	49.61	0.0000
Year collected	1	0.0008	0.11	0.7472	1	0.0666	10.79	0.0041
Inoculum mixture phenotype:year	1	0.0090	1.20	0.2881	1	0.0681	11.03	0.0038
Residuals	17	0.0075			18	0.0062		

Table 2.4. Analysis of variance for virulence of *Zymoseptoria tritici* isolate mixtures (10 isolates each) collected from the North Willamette Valley, Oregon, differing in resistance to azoxystrobin and year of collection, each of two experiments consisted of a factorial of two years of collection (2012 and 2015) and two inoculum mixture phenotypes (azoxystrobin-resistant and sensitive) evaluated in six replicates in the greenhouse.

Source of variation	df	Mean Sq	*F* value	*P* value
Experiment	1	0.3411	47.93	0.0000
Year collected	1	0.0373	5.25	0.0278
Inoculum mixture phenotype	1	0.5397	75.82	0.0000
Experiment:year collected	1	0.0301	4.23	0.0469
Experiment:inoculum mixture phenotype	1	0.0001	0.02	0.8956
Year collelcted:inoculum mixture phenotype	1	0.0158	2.21	0.1452
Experiment:year collected:inoculum mixture phenotype	1	0.0599	8.41	0.0062
Residuals	37			

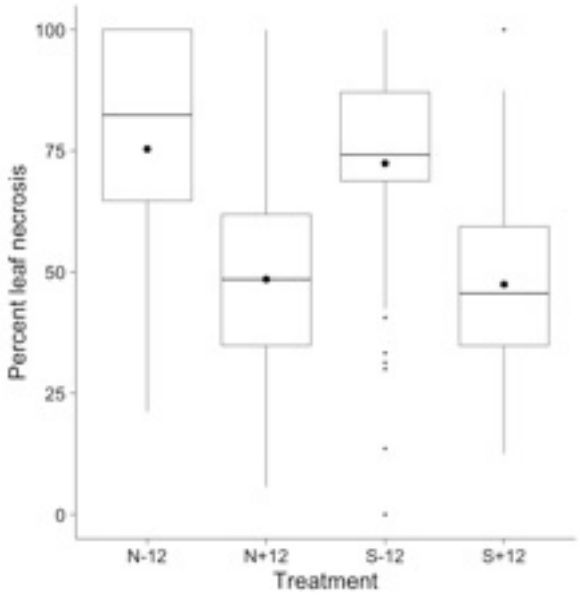

Figure 2.1. Virulence of *Zymoseptoria tritici* isolate mixtures (12 isolates each) collected from the Willamette Valley, Oregon, in 2012 and evaluated in a factorial of two azoxystrobin phenotypes ("-" = sensitive, "+"= resistant) and two source populations ("N"=North, "S" = South). Boxes encompass the second and third quartiles. Within boxes, horizontal lines are medians and dots are means. The whiskers indicate the 1.5-quartile range on either side of the box. Data beyond the ends of the whiskers are outliers and plotted as points.

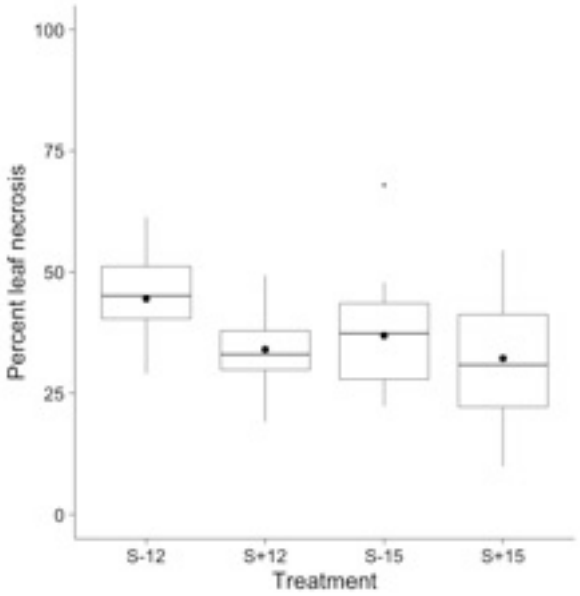

Figure 2.2. Virulence of *Zymoseptoria tritici* isolate mixtures (10 isolates each) collected from the South Willamette Valley, Oregon, and evaluated in a factorial of two azoxystrobin phenotypes ("-" = sensitive, "+"= resistant) and two collection years ("12"=2012, "15" = 2015) in a greenhouse trial. Boxes encompass the second and third quartiles. Within boxes, horizontal lines are medians and dots are means. The whiskers indicate the 1.5-quartile range on either side of the box. Data beyond the ends of the whiskers are outliers and plotted as points.

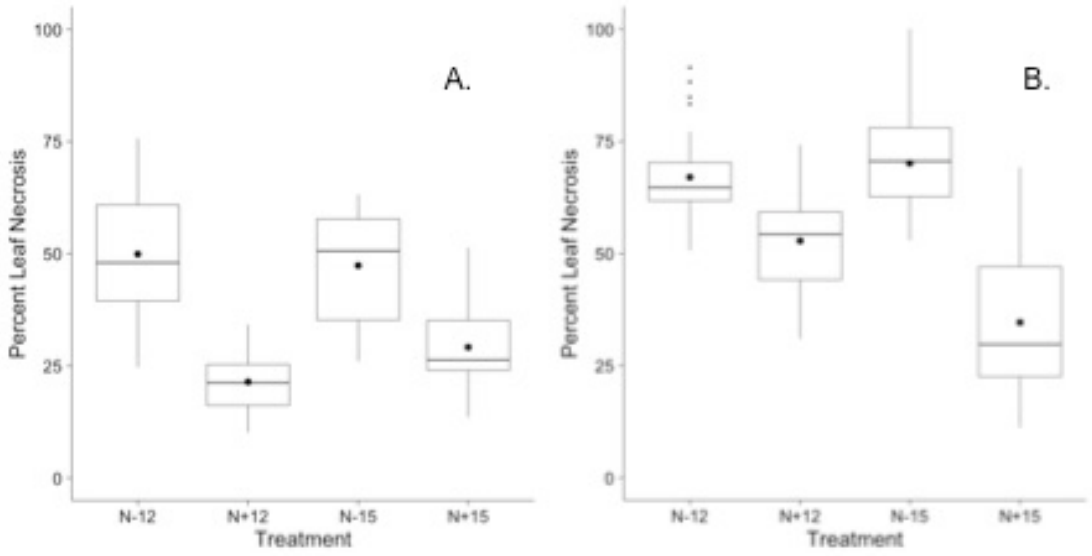

Figure 2.3. Virulence of *Zymoseptoria tritici* isolate mixtures (10 isolates each) collected from the North Willamette Valley, Oregon, and evaluated in a factorial of two azoxystrobin phenotypes ("-" = sensitive, "+"= resistant) and two collection years ("12"=2012, "15" = 2015), in greenhouse experiment 1 (A) and experiment 2 (B). Boxes encompass the second and third quartiles. Within boxes, horizontal lines are medians and dots are means. The whiskers indicate the 1.5-quartile range on either side of the box. Data beyond the ends of the whiskers are outliers and plotted as points.

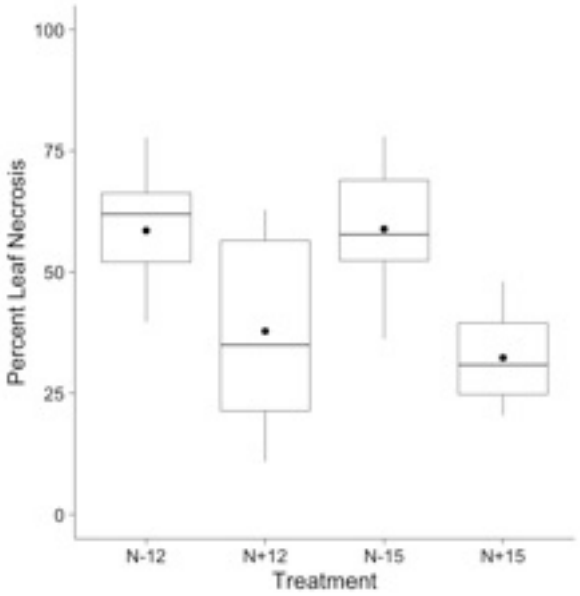

Figure 2.4. Virulence of *Zymoseptoria tritici* isolate mixtures (10 isolates each) collected from the North Willamette Valley, Oregon, and evaluated in a factorial of two azoxystrobin phenotypes ("-" = sensitive, "+"= resistant) and two collection years ("12"=2012, "15" = 2015) in a greenhouse trial. Boxes encompass the second and third quartiles. Within boxes, horizontal lines are medians and dots are means. The whiskers indicate the 1.5-quartile range on either side of the box. Data beyond the ends of the whiskers are outliers and plotted as points.

CHAPTER 3: VARIABLE FITNESS EFFECTS OF FUNGICIDE RESISTANCE IN FIELD EXPERIMENTS WITH A PLANT PATHOGENIC FUNGUS

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ABSTRACT

Classic evolutionary theory suggests that mutations associated with antimicrobial resistance will come at a fitness cost in the absence of the selective antimicrobial agent. Experimental evidence for fitness costs associated with resistance to anti-microbial compounds is numerous across many biological disciplines, including human pathology, entomology, plant sciences, and plant pathology. In contrast, mixed results of neutral and increased fitness associated with resistance are also present in the literature, where the effect of a given resistance mutation depends on environmental and biological factors. We used *Zymoseptoria tritici,* a model evolutionary plant pathogenic fungus, to test the fitness and competitive ability of fungal isolates with a fungicide resistantce mutation. Large-scale inoculated experiments occurred *in vivo,* in the field. We found a significant change in the frequency of fungicide resistance over time in all four experiments. The direction and magnitude of these changes, however, differed by experimental location, year of experiment, and inoculum resistance treatment (fungicide-resistant, resistant/sensitive mixture, and fungicide-sensitive). Overall, it seems that the competitive ability of resistant isolates varied depending upon environmental conditions, possibly including initial resistance frequency.

INTRODUCTION

Experimental evidence for fitness costs associated with resistance to anti-microbial compounds is numerous across many biological disciplines. In human pathology, most early laboratory studies suggested that antibiotic resistance mutations incited a fitness cost (reviewed by Andersson and Levin, 1999). In entomology,

examples include fitness trade-offs associated with insecticide resistance in bollworms (*Pectinophora gossypiella*) and leafrollers (*Choristoneura rosaceana*) (Carrière et al. 1994; Higginson et al. 2005). In plant sciences, many studies link herbicide resistance alleles with overall fitness costs (reviewed by Vila-Aiub et al., 2009). In the field of plant pathology, examples include pathogenicity disadvantages associated with fungicide-resistant rice blast (*Magnaporthe oryzae*), grey mold (*Botrytis cinerea*), and Septoria leaf blotch (*Zymoseptoria tritici*) pathogens (Karaoglanidis, Thanassoulopoulos, and Ioannidis 2001; Ma and Uddin 2009; Hagerty and Mundt 2016).

While examples of clear fitness trade-offs associated with resistance mutations are common in the literature, mixed results are also present, where the effect of a given resistance mutation depends on many factors (Chen et al. 2007; Karaoglanidis, Luo, and Michailides 2010). For example, associations between plant pathogen fitness and resistance to strobilurin fungicides can be highly variable even with the same resistance mutation (Avila-Adame and Köller 2003; Chin et al. 2001; Karaoglanidis, Thanassoulopoulos, and Ioannidis 2001; Ma and Uddin 2009; Hagerty and Mundt 2016). In addition, the effect and depth of the fitness penalty of herbicide resistance depends on many factors, including the environment, the genetic background in which the mutation occurs, and the specific mutation conferring resistance (Bergelson and Purrington 1996; Délye et al. 2013; Menchari et al. 2008; Vila-Aiub, Neve, and Powles 2009). Furthermore, the insensitivity to antimicrobials in human pathogens has been found to be much more complex than the standard "fitness cost of resistance" theory (Singer, Ward, and Maldonado 2006; Davies and

Davies 2010; Beceiro, Tomás, and Bou 2013; Skurnik et al. 2013). For example, while recently emerged resistant individuals may have a fitness cost, costs are likely to be ameliorated by subsequent evolution via compensatory mutations (Andersson and Levin 1999). Compensatory mutations have the capacity to restore fitness disadvantages either in the presence or absence of the antimicrobial agent (Wiesch, Engelstädter, and Bonhoeffer 2010). Further, positive associations between antimicrobial resistance and pathogen virulence and/or fitness have recently been found in a number of human pathogens (Singer, Ward, and Maldonado 2006; Davies and Davies 2010; Beceiro, Tomás, and Bou 2013).

Highly controlled, *in vitro* experiments to test fitness tradeoffs with resistance mutations are numerous. However, large-scale, multi-year, *in vivo* experiments in the presence of environmental variation to measure fitness tradeoffs associated with resistance mutations are lacking in the literature. This absence is likely due to challenges that come along with experimental complexity and scale. Understanding the fitness trade-off dynamic on a large scale, in the presence of environmental variation, will allow us to make more accurate predictions about the evolutionary trajectory of mutations in a population.

We utilized *Zymoseptoria tritici,* the causal agent of Septoria tritici blotch (STB) of wheat as a model to explore fitness costs of a fungicide resistance mutation on a large agricultural scale. Generally, *Z. tritici* is considered a well-established model organism for the study of population dynamics and evolution (Dean et al. 2012). *Z. tritici* is a heterothallic ascomycete fungus with a dominant sexual stage of aerially dispersed ascospores (Ponomarenko, Goodwin, and Kema 2011) and causes

localized necrotic lesions on foliage. Ascospore showers begin in autumn and result in high diversity and a large effective population size (Linde, Zhan, and McDonald 2002). As the winter rains ensue, STB transitions from the sexual stage to multiple generations of splash-dispersed asexual conidia (Ponomarenko, Goodwin, and Kema 2011). The asexual dispersal is limited to a few meter radius (Zhan, Mundt, and McDonald 2001), allowing us to study isolated populations in field plots without risk of contamination. *Z. tritici* is a hemi-biotroph, and infection begins as a chlorotic lesion on a green wheat leaf. Then, *Z. tritici* slowly transitions to a necrotic lesion as the pathogen transitions to necrotrophic phase later in the disease cycle (Ponomarenko, Goodwin, and Kema 2011). Pycnidia, asexual fruiting bodies, form within the lesion and are often organized in rows, filling the substomatal spaces. The asexual latent period depends on temperature, leaf wetness, and precipitation but generally is 20 ± 4 days depending on environmental conditions (Henze et al. 2007). STB is one of the top yield-limiting diseases of wheat in Europe (Torriani et al. 2015), and in the Willamette Valley of Oregon (Mundt et al. 1999), where high fungicide input along with high rainfall provides favorable conditions for the development of fungicide resistance.

Fungicide use to control STB places strong directional selection for fungicide resistance, and favors a rapid increase in the frequency of fungicide-resistant phenotypes. Strobilurin fungicides to control STB were introduced in the early to mid 2000s and, since their relatively recent introduction, the G143A single base pair mutation for complete strobilurin resistance has been detected in the United Kingdom, United States, Czech Republic, Morocco, Ireland, UK, France, Germany,

and Denmark (Gisi, Pavic, Stanger, Hugelshofer, Sierotzki, et al. 2005; Fraaije et al. 2005; Drabešová et al. 2013; Estep et al. 2013; Siah et al. 2014). A high level of resistance to azoxystrobin is conferred by the G143A mutation in the mitochondrial gene encoding cytochrome B, where a single base pair mutation at codon 143 substitutes glycine by alanine (Gisi et al., 2002; Fraaije et al., 2005).

To test fitness costs of resistance, we supplemented naturally occurring populations of Z. tritici in replicated wheat plots at two locations in Oregon to attain different proportions of initial strobilurin resistance. Inoculated treatments included: fungicide-resistant isolates with the G143A mutation, fungicide-sensitive isolates without the G143A mutation, and 50/50 resistant/susceptible isolate mixtures. After successful inoculations, we monitored the persistence and competitive ability of the G143A resistance mutation over time. We found that the fitness and persistence of the G134A mutation may depend on location, year, and initial frequency of resistance.

MATERIALS AND METHODS

Experiment Location and Plot establishment. Field plots of the winter wheat variety, 'Skiles', were established during the 2013-2014 and 2014-2015 growing seasons at the Oregon State University (OSU) Botany and Plant Pathology Field Laboratory (44.568974, -123.242502), the "Botany Farm", and the OSU Hyslop Field Laboratory (44.634638, -123.196917), the "Hyslop Farm" in Corvallis, OR. The Botany Farm is located approximately 11 km south of the Hyslop Farm. The Botany Farm is in close proximity to the city of Corvallis, is generally surrounded by long-term grass seed rotations, and typically hosts row crop and fruit tree trials with very limited wheat planted annually. The Botany Farm's location, proximity to a city, and

on-farm practices limit fungicide exposure. The Hyslop Farm is generally surrounded by more commercial wheat acreage and hosts far more wheat trials than the Botany Farm. Therefore, the Hyslop Farm presumably receives higher fungicide exposure than the Botany Farm. Most soils of Hyslop Farm are classified as Woodburn silt loam; most soils of the Botany Farm are classified as Chehalis silty clay loam.

The experiment consisted of seven treatments, including non-inoculated control plots. Each treatment was assigned randomly to each of seven replicate plots at both experimental locations and in both years of the study. Equal sized plots of wheat and barley, variety 'Alba' were alternated in a checkerboard pattern so that the barley provided a buffer between the wheat plots (Supplemental Figure 3.1). Plots were planted on 24 October 2013 at Hyslop and 25 October 2013 at Botany Farm and on 9 October 2014 at Hyslop Farm, and 10 October 2014 at Botany Farm. Experiments in the 2013-14 and 2014-15 wheat seasons are hereafter referred to as 2014 and 2015, respectively. Plots received a balanced starter fertilizer (28 kg/ha of N, P_20_5, and K_20) before planting and 135 kg N/ha at late tillering. Weed control was via appropriate herbicides. Randomized complete block designs were utilized at Hyslop in the 2014 and 2015 experiments and in the Botany Farm 2014 experiment, with blocks running parallel, east to west. Due to field availability for the 2015 experiment at the Botany Farm, blocks were oriented in a pattern to avoid areas of the field subject to winter flooding.

Isolate Selection and Inoculations. Plots were inoculated with isolates collected in 2012 and originating from two source populations from the Willamette Valley, Oregon. The collections were genotyped in 2012 and are described by (Estep

et al. 2015). Brief methods from Estep et al. (2015) include sampling uppermost leaves naturally infected by *Z.tritici* during 2012 from fungicide trials. Two trials in the Willamette Valley were sampled, one trial was within a commercial wheat field in the North Willamette Valley, and the other trial was at the Hyslop Field Laboratory in the South Willamette Valley. Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) protocols were used to screen isolates in the collections for the presence or absence of the G143A mutation for complete resistance to azoxystrobin. Prior sequence analysis of the cytochrome *b* gene revealed that all resistant isolates carried a mutation resulting in the replacement of glycine by alanine at codon 143 (G143A) (Fraaije et al. 2005).

From the isolates collected and genotyped by Estep et al. (2015), we selected 12 isolates with the G143A mutation and 12 isolates without the G143A mutation from each of the source populations, North Valley and South Valley. These isolates (48 in total) were used to make six inoculum mixture treatments in a factorial design (Table 3.1). These treatments were applied to alter the proportion of resistance in the naturally occurring populations. Using populations originally isolated from two different source populations enabled us to evaluate consistency of the different inoculum treatment effects.

Field Inoculum Preparation. Estep et al. (2012) placed isolates in long-term storage in 50% glycerol in a -80°C freezer. Thirteen days before scheduled field inoculations, we removed isolates from the freezer and transferred them onto yeast-malt-sucrose agar (YMA – 15g agarose, 4g sucrose, 4g malt extract, 4g yeast extract, 1L DI H_20) Petri plates. After three days of growth, isolates were transferred onto

fresh YMA plates and allowed to grow for three additional days. Seven days prior to field inoculations, secondary transfers were rinsed with sterile deionized water into 250ml Erlenmeyer flasks containing 150ml of sterile malt-sucrose broth (20g sucrose, 20g malt extract, 5g yeast, 1L DI water). Each flask contained a single *Z. tritici* isolate (48 flasks total). Flasks were plugged with sterile foam, capped with aluminum foil, and shaken ("Orbit", Labline Instruments, India) at 0.8 revolutions per second on the lab bench top for seven days. After seven days of benchtop shaking, the evening before scheduled field inoculations, isolates were blended (Waring Laboratory Science, City, State) individually for 30 seconds and subsequently strained with four layers of cheesecloth. Inoculum treatments were mixed and diluted with DI water to achieve a spore concentration of $2x10^6$ and placed in cold storage until the following morning. On the morning of field inoculations, inoculum was transferred into 7.57L "Green Thumb" (True Value, Chicago, IL) garden sprayers. Each sprayer was filled with a different inoculum treatment (six sprayers total), and the sprayers were used to apply inoculum to field plots on 25 February 2014 at a rate of 250 ml of $2x10^6$ *Z. tritici* inoculum per plot. The Botany Farm inoculation was unsuccessful due to unexpected, intense afternoon sun exposure at the time of inoculation and was thus repeated on 24 March 2014. To help increase the success of the inoculation, plots were covered with 3-mil black plastic for approximately 67 hours after inoculation and was highly successful.

Due to challenging factors that contribute to the success of *Z. tritici* field inoculations that became apparent in 2014, we conducted two "back-to-back" inoculations in 2015, separated by six days. In addition to the double inoculation, all

plots were covered with 3 mil black plastic tarps for 48 hours. Both inoculations were successful.

Sampling, isolating, and quantification of azoxystrobin resistance. After successful inoculations, 12 uppermost leaves in the canopy with a *Z. tritici* lesion showing obvious pyncidia were collected uniformly over the plots in one early season collection, "Collection 1", and one late season collection, "Collection 2". In each year, Collection1 and Collection 2 were separated by two *Z. tritici* generations, based upon growing degree calculations (Henze et al. 2007). After leaves were collected, we followed identical *Z. tritici* isolation procedures as described by Hagerty et al. (submitted). Isolates selected for the study were blindly phenotyped for qualitative azoxystrobin fungicide resistance. To perform phenotyping, isolates were transferred onto two petri plates – one plate with non-amended YMAa and one plate with YMAa amended with 1ppm technical grade azoxystrobin (Syngenta, Basel, Switzerland). Three days after plating, isolate growth on the azoxystrobin plate was compared to that of the non-amended plate. Isolates with the G143A mutation were scored at 100% accuracy.

Collection validations and farm baseline. Additional isolates were collected in April 2014 from wheat breeding populations planted at both the Botany Farm (71 isolates) and Hyslop Farm (99 isolates) in order to validate our estimates of strobilurin resistance in non-inoculated plots of the experiments.

STATISTICAL ANALYSIS

All analyses were performed in R (version 3.1.2, 2014-10-31) using the R studio graphical user interface (GUI) (Version 0.98.1091 RStudio, Inc.). The *glmer*()

function in the *lme4* package was used to build generalized linear mixed models. We utilized generalized linear models with a binomial response variable to evaluate both treated plots and controls. Eight separate analyses occurred for each of the experimental locations and years (Botany Farm 2014, Botany Farm 2015, Hyslop 2014, Hyslop 2015), for both treated and control plots. To arrive at the final generalized linear model for each experiment, we conducted a series of model comparisons to test for significance of the terms included in the model. With the proportion of resistant isolates as the dependent variable in all potential models, we chose from the following contending models differing only by the explanatory variable(s): model 1) a null model; model 2) inoculation treatment; model 3) collection time; additive model 4) inoculum treatment + collection time; interactive model 5) inoculum treatment * collection time. We chose the contending models to encompass any changes in resistance due to inoculum treatments and the collection time. Each model under consideration was reasonably supported by the data, and met assumptions of generalized linear models. To rank the models under consideration we performed a drop in deviance chi-square test and a threshold of $p>0.05$ to determine which models were best supported.

Non-inoculated control plots were analyzed separately from inoculated plots because controls were included in the study to confirm the success of the inoculations, and understand the dynamics of natural inoculum. Controls did not serve as a direct statistical comparison to inoculated plots. To evaluate control plots, the model included only the collection time as the explanatory variable, with

proportion of resistant isolates collected as the response variable. Collection time occurred at two levels: Collection 1 and Collection 2.

To evaluate the inoculated plots, the explanatory variables in the model included collection time, inoculum source, and inoculum resistance, and all possible interactions between main effects with the proportion of resistant isolates collected as the response variable. Collection time occurred at two levels: Collection 1 and Collection 2; inoculum source occurred at two levels: North Valley and South Valley; inoculum resistance occurred at three levels: positive, negative, and mixture.

Analyses combined over experiments indicated significant location (Hyslop Farm/Botany Farm) by year (2014/2015) interactions. Therefore, data of the four experiments were analyzed separately. The inoculum source (North Valley vs. South Valley) and inoculum resistance (resistant, mixture, sensitive) interaction was not significant in any of the four experiments; therefore we excluded the inoculum source term from the model and consolidated the treatments to consider only the inoculum resistance status of resistant, mixture, and sensitive, regardless of source location.

Planned comparisons. Using the models, the following planned comparisons were made to quantify the persistence and competitive ability of the azoxystrobin-resistant phenotype in inoculated plots: resistant inoculated plots in Collection 1 vs. Collection 2, mixture inoculated plots in collection 1 vs. collection 2, sensitive inoculated plots in collection 1 vs. collection 2. Planned comparisons were made independently for each of the four experiments (Table 3.3).

RESULTS

Isolates collected from field plots in 2014 indicated high consistency in estimating the proportion of azoxystrobin resistance. At the Botany Farm, nine of 71 (12.7%) isolates sampled from breeding plots on the same farm early in the season scored resistant to azoxystrobin, while the control plots of the experiment averaged 11.2% in Collection 1 (Figure 3.1). At the Hyslop Farm, 66 of 94 (70.2%) isolates sampled from breeding plots early in the season scored resistant to azoxystrobin, while the control plots in the experiment sampled averaged 70.3% in Collection 1 (Figure 3.1).

As validated with model selection procedures, the inoculation treatments were successful in establishing differing levels of initial frequencies of azoxystrobin resistance. Inoculations with resistant, sensitive, and a mixture of resistant/sensitive isolates always showed the highest, lowest, and intermediate levels of resistance among the four inoculations treatments, respectively. There was a larger range in resistance frequencies among inoculation treatments in 2015, a year in which we had developed improved inoculation procedures. Botany Farm 2015 and Hylsop Farm 2014 control plots did not change over time from Collection 1 to Collection 2 (Table 3.2). We do have weak evidence that Botany Farm 2014 control plots decreased from Collection 1 to Collection 2, and strong evidence that Hyslop Farm 2015 plots increased from Collection 1 to Collection 2 (Table 3.2).

Drop in deviance chi-square tests for model comparisons revealed additive main effects of collection time and inoculation treatment at Botany Farm experiments, and interactive effects of collection time and inoculation treatment at Hyslop Farm.

In the inoculated plots, there is a significant effect of time and treatment in all four experiments (Table 3.3). At the Botany Farm, the effect of time did not depend on treatment, and resistance decreased in both years from Collection 1 to Collection 2, regardless of treatment. At the Hyslop Farm the effect of time depended on the treatment in both years. At the Hyslop Farm in 2014 resistance mixture treatments and sensitive inoculated treatments decreased in resistance over time, whereas resistant inoculated plots did not change over time. Resistance was favored at Hyslop Farm experiments in both inoculated and control plots; sensitivity was favored at Botany Farm experiments in both inoculated and control plots. At the Hyslop Farm in 2015, sensitive inoculated plots increased in resistance over time; whereas resistant inoculated plots did not change over time (Table 3.3). The significant time and treatment interaction at Hyslop Farm experiments seems to be driven by the resistant inoculum treatment, which attained high initial frequency and changed little over time.

DISCUSSION

The null hypothesis for this study is dictated by evolutionary and ecological theory suggesting that resistance mutations impart fitness trade-offs. We tested this hypothesis utilizing *Z. tritici* strobilurin fungicide resistance in repeated experiments under a variety of field environments. We found a significant change in the frequency of fungicide resistance over time in all four experiments. The direction and magnitude of these changes, however, differed by experimental location, year of experiment, and inoculum resistance treatment (fungicide-resistant, resistant/sensitive mixture, and fungicide-sensitive). Overall, it seems that the competitive ability of resistant isolates

varied depending upon environmental conditions, possibly including initial resistance frequency.

Fitness associated with fungicide resistance in the control plots differed between the two years of the study. At the Botany Farm, resistance declined significantly over time in 2014, but not 2015. At the other experimental site, there was essentially no change in resistance frequency over the two generations of study in 2014, but resistance frequency increased significantly over time in 2015. These results may simply be a random effect of year. Alternatively, there may have been selection for compensatory mutations that increased the fitness of genotypes containing the fungicide resistance mutation.

Differences in baseline resistance between Botany Farm and Hyslop Farm are likely a result of the location of each experimental site and the surrounding agricultural practices. Artificial inoculations were used to alter the initial frequency of fungicide resistance among treatments at the two sites. We used mixtures of isolates in an attempt to average potential fitness effects of resistance over multiple genetic backgrounds, and we found that isolate mixtures collected from two different regions of the Willamette Valley did not differ in their effects on pathogen fitness. Because we inoculated the same treatments at locations with different levels of background resistance, the range of initial frequencies differed between the two experimental sites. Despite these substantial differences in baseline level of fungicide resistance between the two experimental locations, we have great certainty that our inoculations successfully established different levels of initial resistance frequency. First, in all four experiments, the proportion of resistant isolates collected in each treatment group

during the first collection matched the order of the proportion of resistant isolates in the corresponding inoculum (i.e. resistant inoculated highest, mixture inoculated second highest, and sensitive inoculated lowest proportion of resistance). For all treatments, it appeared that deviations from inoculum resistance observed at the first collection time were generally in the direction of the baseline resistance for the experimental location, demonstrated by the control plots.

The results of this study paint a complex picture in regards to the fitness trade offs associated with resistance mutations. Most prior studies have reported either a positive or negative main effect of resistance mutations on fitness in the absence of fungicide selection pressure. Our results indicate that the effect of the resistance mutation is in fact very dependent on local variables. In both Botany Farm experiments, where the baseline level of resistance in natural inoculum was low, all inoculated treatments decreased in fungicide resistance over time from Collection 1 to Collection 2, and the decrease in resistance was not dependent upon treatment. Results from the Botany Farm experiments are indicative of a fitness trade off associated with resistance because the resistant phenotype reduced in frequency in the absence of fungicide selection pressure. In contrast, results from the Hyslop Farm are dependent upon a treatment by time interaction. While resistant treatments at Hyslop Farm had no change in resistance frequency over time in either 2014 or 2015, the effect of time differed for mixture and sensitive inoculated experiments. This study compared fitness of sensitive and resistant isolates under field conditions, where many environmental variables affect the survival and reproduction of isolates. This increase in environmental variables may have caused more interactive effects than

are observed in studies that measure fitness in a greenhouse or *in vitro*. Large scale, costly, but realistic, trials must be conducted in addition to more controlled experiments to fully understand fitness trade-offs.

In addition to putative environmental variation affecting the competitive ability of fungicide-resistant *Z. tritici,* we have preliminary evidence of frequency dependent selection. When the resistant phenotype was at high frequency in the population, fitness of the resistant phenotype seems to have been neutral or favored. Both resistant treatments in 2014 and 2015 at Hyslop were established at a high level of resistance (greater than an average of 80% resistant isolates) and plots maintained the high level of resistance over time from Collection 1 to Collection 2. These are the only two treatments that stayed stable over time. With the high background level of fungicide resistance at Hyslop, as established by sampling non-inoculated control plots (Figure 3.2), this pattern suggests a positive frequency dependent selection favoring fitness of the resistant phenotype when it is more common in the population. This result is consistent with our survey of commercial wheat fields throughout the Willamette Valley conducted at 6-month intervals from June 2014 to January 2016 (Hagerty et al. submitted). During this survey period, the mean azoxystrobin resistance increased from 63 to 93% valley-wide. Importantly, azoxystrobin resistance increased even over the winter months, when no fungicides were applied, as well as in fields that received no strobilurin applications. Thus, there now appears to be a fitness advantage associated with resistance in commercial fields, even in the absence of fungicide application, perhaps driven by selection for fitness modifiers or adapted gene complexes in a population that initially became predominately

fungicide-resistant via selection driven by application of strobilurin fungicides when these chemicals were still effective.

Our experiments provide only the mean phenotypic frequency of fungicide resistance in each plot over time, as resources were unavailable to track the frequency of individual, inoculated isolates with molecular markers. As most naturally occurring isolates are different genotypes (Zhan, Mundt, and McDonald 2001), it would have been impossible to follow individual genotypes of naturally occurring inoculum. As a result, we could not determine the relative contributions of artificially inoculated versus naturally occurring genotypes to the total phenotypic change that was measured. Nonetheless, the design of the experiments allow for some relevant comparisons. For example, the resistant inoculum treatment contained the same 12 resistant isolates as in the mixture inoculum treatment. Thus, temporal stability of azoxystrobin resistance at high frequency in the Hyslop Farm experiments cannot be explained simply by a high fitness of these isolates in that environment, as the mixture treatment contained the same 12 resistant isolates inoculated into the same background population, yet resistance decreased over time in the mixture treatment.

Fungicide resistance of *Z. tritici* allowed us to efficiently test hypotheses on a field scale. However, this study has several limitations for making broader conclusions about ecological and environmental theory. On an experimental level, a major limitation was the time scale of experiment, which spanned two generations of the pathogen within one growing season, but was not able to measure the ability of isolates to survive overwintering. We studied resistance dynamics in replicated field plots, but were unable to capture any differences that may occur if dynamics are

studied at the landscape scale. Finally, we assumed that the differences in fitness between the resistant and susceptible isolates were due to the G143A mutation, but the genetic background of these isolates may have also had a fitness effect. In an effort to ameliorate this effect, we utilized inoculum mixtures because there should be fewer differences in the genome not associated the G143A mutations when averaged over multiple isolates.

Our results may have relevance to other organisms and to other selective agents, such as antibiotics, insecticides, herbicides, and other fungicides. Similarities between animal and plant diseases have become increasingly apparent at both the molecular (Staskawicz et al. 2001; Ausubel 2005; Sexton and Howlett 2006) and ecological (Mundt et al. 2009; Borer et al. 2011) levels. Some antimicrobials are used for the control of both human and plant diseases (Verweij et al. 2009). Further, as with human diseases (Singer, Ward, and Maldonado 2006; Davies and Davies 2010; Beceiro, Tomás, and Bou 2013), there is evidence for a positive association between fungicide resistance and fitness in some cases (Kim and Kim, 2009; Yang et al., 2013; Köycü et al., 2014). It thus seems logical that associations between antimicrobial insensitivity and fitness discovered for human diseases may also be highly relevant to plant diseases, and vice versa.

We have found evidence for a strong environmental component potentially driving the competitive ability of resistant isolates. Results of this study have implications for how resistance mutations might persist in a population and thus change the evolutionary potential of an organism. Although persistence of a mutation is

dependent upon fitness, it seems the fitness of the mutation may depend strongly on environmental effects.

Table 3.1. Factorial design of two inoculum sources (North and South Valley) and three fungicide resistance (fungicide-resistant, fungicide-sensitive, resistant/sensitive mix) inoculum types resulting in six unique treatment groups for *Zymoseptoria tritici* field inoculations in the Willamette Valley, Oregon.

		Inoculum source	
		North Valley	South Valley
Inoculum resistance	Resistant (G143A+)	12 isolates	12 isolates
	Sensitive (G143A-)	12 isolates	12 isolates
	Mix (G143A +/-)	24 isolates	24 isolates

Table 3.2. Generalized linear model summaries for non-inoculated control plots that were evaluated for the proportion of resistant *Zymoseptoria tritici* isolates at two collection times in four experiments (Botany Farm 2014, Botany Farm 2015, Hyslop Farm 2014, and Hyslop Farm 2015).

Botany Farm 2014

Coefficients:	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-0.23	1.34	-0.17	0.862
Collection Time	-1.69	1.10	-1.53	0.126

Botany Farm 2015

Coefficients:	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-1.31	0.82	-1.60	0.109
Collection Time	-0.13	0.51	-0.25	0.800

Hyslop Farm 2014

Coefficients:	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	1.23	0.70	1.75	0.081
Collection Time	-0.23	0.47	-0.48	0.634

Hyslop Farm 2015

Coefficients:	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-0.28	0.66	-0.43	0.670
Collection Time	1.29	0.47	2.72	0.007

Table 3.3. Using a generalized linear model and subsequent contrast statements, planned comparisons were made to assess the competitive ability of the azoxystrobin-resistant phenotype in inoculated plots over time from Collection 1 to Collection 2. For experiments conducted at the Botany Farm one contrast of Collection 1 versus Collection 2 was conducted because there was no interaction between time and inoculation treatment. For experiments conducted at the Hyslop Farm, there is a time by treatment interaction, and therefore three contrasts were conducted and included: resistant inoculated plots in Collection 1 vs. Collection 2, mixture inoculated plots in Collection 1 vs. Collection 2, and sensitive inoculated plots in Collection 1 vs. Collection 2. Comparisons were made independently for four experiments (Botany Farm 2014, Botany Farm 2015, Hyslop Farm 2014, and Hyslop Farm 2015).

Botany Farm 2014

Contrast	Estimate	Std. Error	X^2 value	DF	Pr(>|X^2|)
Collection 2 - Collection 1	-3.343	1.043	10.277	1	0.001

Botany Farm 2015

Contrast	Estimate	Std. Error	X^2 value	DF	Pr(>|X^2|)
Collection 2 - Collection 1	-2.192	0.294	55.638	1	0.000

Hyslop Farm 2014

Contrast	Estimate	Std. Error	X^2 value	DF	Pr(>|X^2|)
Collection 2 - Collection 1: resistant	-0.063	0.412	0.023	1	0.879
Collection 2 - Collection 1: mix	-0.814	0.261	9.703	1	0.002
Collection 2 - Collection 1: sensitive	-2.990	0.554	29.120	1	0.000

Hyslop Farm 2015

Contrast	Estimate	Std. Error	X^2 value	DF	Pr(>|X^2|)
Collection 2 - Collection 1: resistant	-0.671	0.561	1.429	1	0.232
Collection 2 - Collection 1: mix	0.347	0.242	2.059	1	0.151
Collection 2 - Collection 1: sensitive	0.687	0.267	6.593	1	0.010

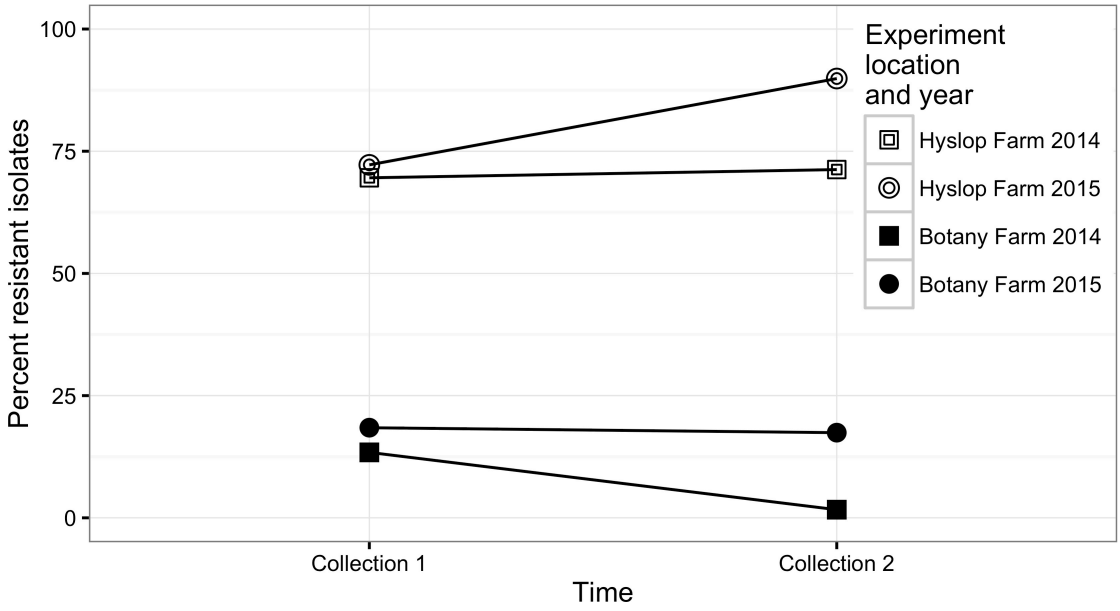

Figure 3.1. Percentage of azoxystrobin-resistant *Zymosetptoria tritici* isolates sampled from non-inoculated control plots in four different experiments: Hyslop Farm 2014 (open square), Hyslop Farm 2015 (open circle), Botany Farm 2014 (closed square), Botany Farm 2015 (closed circle). Isolates were recovered from the plots in Collection 1 in early spring, and Collection 2 in late spring, span of two pathogen generations, to observe the change in frequency of the azoxystrobin-resistant *Z.tritici* phenotype over time.

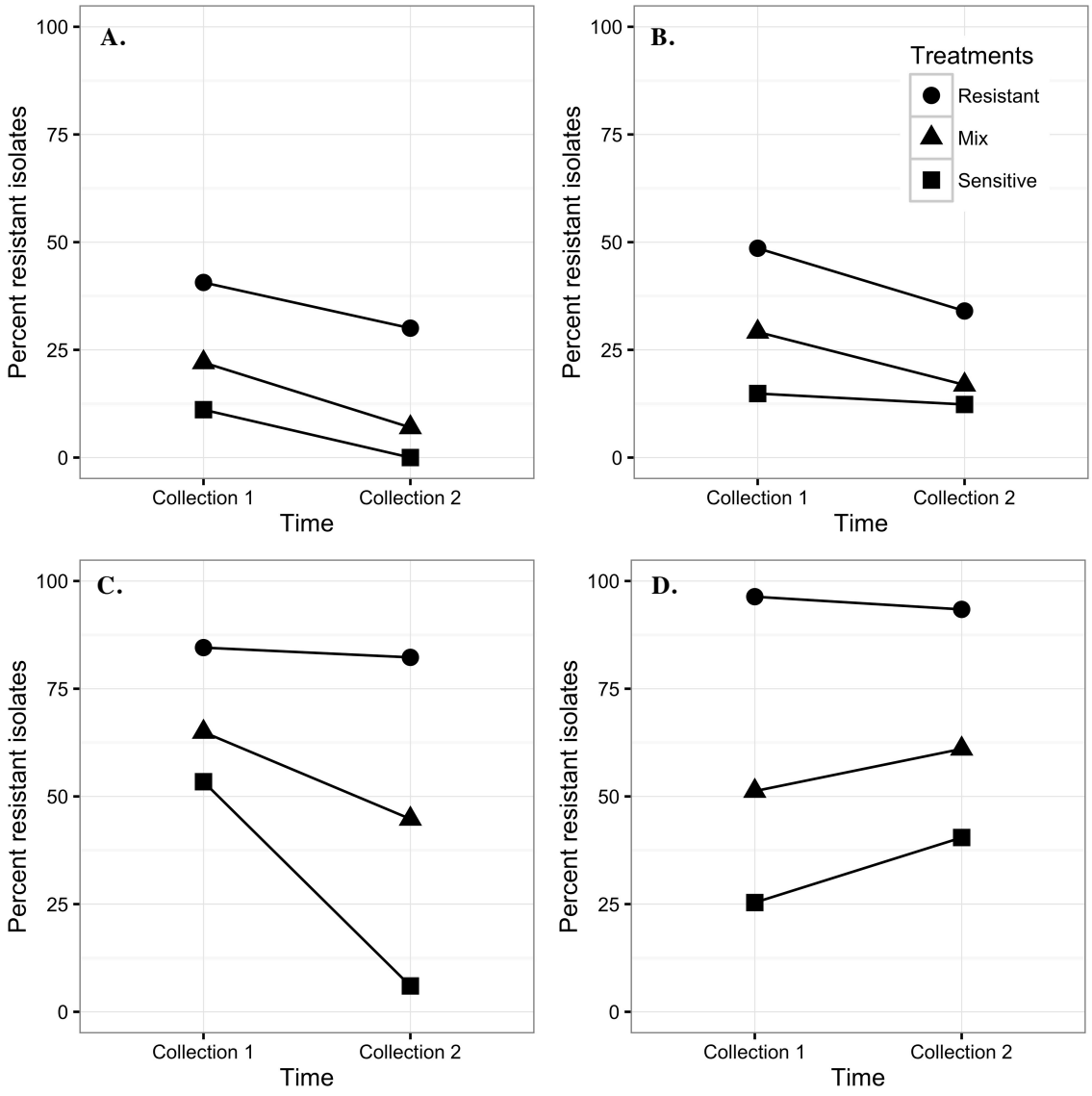

Figure 3.2. Percentage of azoxystrobin-resistant *Zymosetptoria tritici* isolates sampled from inoculated plots in four different experiments: Botany Farm 2014 (A), Botany Farm 2015 (B), Hyslop Farm 2014 (C), Hyslop Farm 2015 (D). Plots were inoculated with a mixture of 12 azoxystrobin-resistant isolates (circle), a mixture of six azoxystrobin-resistant isolates and six azoxystrobin-sensitive isolates (triangle), or a mixture of twelve azoxystrobin-sensitive isolates (square). After successful

inoculations, isolates were recovered in the first *Z.tritici* generation (Collection 1), and the third *Z.tritici* generation (Collection 2) to observe the change in frequency of the azoxystrobin-resistant *Z.tritici* phenotype over time.

Supplemental Figure 3.1. Aerial view of the experimental design at the Oregon State University Botany Farm in Corvallis, Oregon. Dark rectangles are the *Zymoseptoria tritici* inoculated 1.5m x 6m wheat plots, surrounded by barley buffers. Map data © 2016 Google.

CHAPTER 4: TEMPORAL DYNAMICS AND SPATIAL VARIATION OF *ZYMOSEPTORIA TRITICI* FUNGICIDE RESISTANCE: A HIGHERARCHICAL SURVEY OF COMMERCIAL WINTER WHEAT FIELDS IN THE WILLAMETTE VALLEY, OREGON

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ABSTRACT

Fungicide resistance can cause disease control failure in agricultural systems, and is particularly concerning with *Zymoseptoria tritici*, the causal agent of Septoria triciti blotch of wheat. In North America, the first QoI resistant *Z. tritici* was discovered in the Willamette Valley of Oregon in 2012, which prompted this hierarchical survey of commercial winter wheat fields to monitor for azoxystrobin- and propiconazole-resistant *Z. tritici*. Four surveys were conducted in June 2014, January 2015, May 2015, and January 2016. The survey was organized in a hierarchical scheme: regions within the Willamette Valley, fields within the region, transects within the field, and samples within the transect. Overall, azoxystrobin resistance increased from 63 to 93% of isolates sampled as resistant to azoxystrobin from June 2014 to January 2016. Resistance to azoxstrobin increased over time even within fields receiving no strobilurin applications. Propiconazole sensitivity varied among isolates, but stayed relatively stable over time. Sensitivity of both fungicides showed no regional aggregation within the Willamette Valley. Greater than 80% of spatial variation in fungicide sensitivity was at the smallest hierarchical scale of the survey for both fungicides, and the resistance phenotypes were randomly distributed within sampled fields. Results suggest a need for a better understanding of the dynamics of fungicide resistance at the landscape level.

INTRODUCTION

Fungicides are an important contributor to global food production and food security (Gianessi and Reigner 2006; Carvalho 2006; Van Den Bosch et al. 2014). The development of fungicide resistance can result in disease control failure in an

agricultural system, which can lead to reduced crop management options and yield loss. The loss of fungicides to resistance has the capacity to threaten global food security, as 23% of fungicides are sold for use on cereals to control disease(Phillips McDougall 2013). In fact, pathogens of the world's most important staple crops have developed fungicide resistance. Examples include *Rhizoctonia solani* (the causal agent of sheath blight in rice) resistance to jinggangmycin in China (X.R. et al., 2006), *Cercospora sojina* (the causal agent of frogeye leaf spot in soybean) resistance to strobilurins in North America (Zhang, Newman, and Bradley 2012), *Alternaria solani (*the causal agent of early blight in potato) resistance to boscalid in Idaho (Fairchild, Miles, and Wharton 2013), and *Zymoseptoria tritici* (the causal agent of Septoria leaf blotch (STB) of wheat) resistance to strobilurins, triazoles, and succinate dehydrogenase inhibitors (SDHIs) (Gisi, Pavic, Stanger, Hugelshofer, and Sierotzki 2005; Estep et al. 2013; FRAC SDHI Working Group 2013). The rate at which fungicides are being lost due to fungicide resistance may soon exceed the rate at which new fungicides become available to agricultural producers (Van Den Bosch et al. 2014). Furthermore, it is estimated that nine years and 250 M USD are needed to get a new mode of action on the market (Phillips McDougall 2013), not including the stochastic "discovery time" necessary to find a new mode of action.

Pathogen populations with greater genetic diversity and sexual recombination typically have a greater risk for developing fungicide resistance (Linde, Zhan, and McDonald 2002). Regional surveys for fungicide resistance in the highly variable fungus, *Z tritici,* have revealed that many fungicides commonly used to control STB epidemics are no longer effective due to the buildup of resistance in local *Z. tritici*

populations (Drabešová et al. 2013; Siah et al. 2014; Estep et al. 2015). In Europe, strobilurins (quinone outside inhibitor (QoI)) are no longer used for STB control due to complete resistance. Strobilurin resistance in *Z. tritici* is usually conferred by a single base pair mutation in mitochondrial cytochrome B, where at codon 143 glycine is substituted by alanine (Fraaije et al. 2005). This substitution leads to qualitative, or complete resistance. Remaining STB management options in Europe include the triazole family and the SDHI family (Dooley 2015). Triazole fungicides act as demethylation inhibitors (DMI) and interfere with sterol biosynthesis in the fungus (Thompson 1975). Thirteen residues within the CYP51 gene have been identified as subject to mutation (Mullins et al. 2011). Furthermore, the thirteen residues act in concert with 49 possible combinations of mutations identified in the CYP51 gene, giving rise to quantitative resistance (Dooley 2015). Similar to the strobilurins, resistance to SDHIs in *Z. tritici* is qualitative, with a single amino-acid substitution conferring resistance (Skinner et al. 1998). SDHIs have been used in Europe to control STB since 2003, and since their release, monitoring efforts have identified field resistant isolates in France, Germany, Ireland, and England (FRAC SDHI Working Group 2013).

The vast genetic diversity of the *Z. tritici* population (McDonald and Mundt 2016), the substantial local reliance on chemical control, and the ubiquitous presence of STB (Mundt et al. 1999) in the Willamette Valley of Oregon put *Z. tritici* at high risk for developing fungicide resistance. Fungicide use to control STB in the Willamette Valley began in the early to mid-1980s with benomyl. Resistance to benomyl developed quickly (NSF Center for Integrated Pest Management 1999),

prompting the release of propiconazole, marketed as Tilt® (Syngenta Crop Protection LLC, Switzerland), in 1988 (Koepsell et al. 1989). By the early 1990s, approximately one-half of commercial winter wheat fields in the Willamette Valley were sprayed with one application of propiconazole at flag leaf emergence (Feekes 8). By the late 1990s, most fields received a single application of propiconazole annually (Mundt, C. C. unpublished). The first strobilurin fungicide, Quadris® (Syngenta Crop Protection LLC, Switzerland), was registered for wheat in Oregon in 2000 (Pscheidt and Ocamb 2000). Blended products combining a triazole and a strobilurin, Quilt Excel® (Syngenta Crop Protection LLC, Switzerland) were recommended for STB management in 2004 (Pscheidt and Ocamb 2005). In the mid-2000s, the introduction of new aggressive races of stripe rust (*Puccinia striiformis*) (Hovmøller et al. 2008), caused a major change in fungicide spray practices on winter wheat crops in the Willamette Valley. The arrival of new aggressive, temperature-tolerant races of stripe rust to the Willamette Valley were not sufficiently controlled by high temperature, adult plant resistance (Chen 2013) which had been effective at controlling stripe rust in the past. This major population shift forced growers to rely more heavily on fungicides to control stripe rust, with both *P. striiformis* and *Z. tritici* being exposed to the increased fungicide applications. Given the low cost of fungicide applications, as low as approximately 7.50 USD per hectare when tank-mixed with herbicides, three-to-four fungicide applications per year have become standard when wheat prices are high (above 200 USD/Mg) and if rust-resistant varieties are not available.

Studying the emergence and spread of fungicide resistance is difficult because a resistance mutation in a population cannot reasonably be detected until it reaches a

sufficient frequency and, thus, resistance is often not discovered until after major crop loss occurs. As a result, the temporal and spatial patterns of fungicide resistance are not well understood. The emergence of strobilurin resistant *Z. tritici* was first discovered in the United Kingdom in 2002 (Fraaije et al. 2005), and resistance was then detected the following year in Ireland, France, Germany, and Denmark (Gisi, Pavic, Stanger, Hugelshofer, and Sierotzki 2005). (Gisi, Pavic, Stanger, Hugelshofer, and Sierotzki 2005) monitored for resistance to strobilurins and triazoles from 1998 to 2004. No QoI resistant isolates were detected until 2001 but, in 2002, resistance was detected simultaneously in Ireland, United Kingdom, France, Germany, and Denmark. (Drabešová et al. 2013) did not detect azoxystrobin resistance in samples collected prior to 2007 and 100% of isolates sampled were resistant to azoxystrobin at some locations in 2011, coinciding with an increase in local strobilurin fungicide use (Drabešová et al. 2013). (Siah et al. 2014) documented no change in fungicide resistance from 2008 to 2010 at three out of four sites in Morocco. At the fourth site, however, they documented an increase of zero strobilurin resistant isolates in 2008 to 60% of isolates collected resistant to strobilurin in 2010. (McCartney et al. 2007) initiated surveys for azoxystrobin-resistant *Z. tritici* in Ireland in the 2004 and 2005 growing seasons. At the beginning of their study, 45% of isolates sampled were resistant to azoxystrobin before the application of seasonal fungicide sprays. The following year, 87% of isolates sampled were resistant to azoxystrobin.

In contrast to strobilurins, triazoles are more stable, and generally less *Z. tritici* resistance has been detected. A survey effort in Europe tested *Z. tritici* for sensitivity to a range of DMI fungicides, and a slight shift toward less sensitivity was

documented from 2004 to 2005 (Gisi, Pavic, Stanger, Hugelshofer, and Sierotzki 2005). Gradual shifts in triazole sensitivity has yet to result in complete loss of efficacy (Mavroeidi and Shaw 2006; Cools, Fraaije, and Lucas 2005). However, resistant *Z.tritici* isolates have been detected to all members of the triazole class (Cools et al. 2011). Since strobilurins are now ineffective at controlling STB in Europe, the continued heavy reliance on triazoles provide adequate selection pressure that may result in complete loss of triazole efficacy (Cools and Fraaije 2013).

Fungicide resistance of *Z. tritici* in Europe prompted investigation of western Oregon *Z. tritici* populations in 2012. (Estep et al. 2013)) sampled two Willamette Valley populations and discovered the first report of strobilurin resistant *Z. tritici* in North America. At the time of the first sampling (2012), 108 of 261 (41%) isolates were found to be resistant to azoxystrobin. The moderate level of fungicide resistance detected in 2012 can be considered a rare snapshot documenting moderate fungicide resistance before growers and crop consultants noticed loss of strobilurin efficacy. Even so, detecting loss of efficacy with strobilurin can be challenging because this product is typically tank-mixed with other fungicide classes. Subsequently, both molecular data (Estep et al. 2015) and phenotypic data (Hayes et al. 2015) were obtained and results indicated selection for a low level of resistance to propiconazole in the same *Z. tritici* populations.

Little is known about the spatial pattern of fungicide resistance, despite its potential significance for understanding the spread of resistance in agricultural landscapes. One study found fungicide resistance was spatially aggregated for *Botrytis squamosa* in grape vineyards and onion fields (Van der Heyden et al., 2014).

Researchers also found spatial aggregation of fungicide-resistant *Monilinia fructicola* in stone fruit orchards (Elmer, Gaunt, and Frampton 1998). Spatial pattern of fungicide-resistance in *Z. tritici* has not been yet been reported in the literature.

The chemical control of STB in the Willamette Valley of Oregon provides an opportunity to characterize the temporal dynamics and spatial variation of fungicide-resistant *Z. tritici,* given the relatively recent appearance of qualitative resistance to azoxystrobin and quantitative resistance to propiconazole. We monitored *Z. tritici* for resistance to azoxystrobin and propiconazole in the Willamette Valley, with the goal of quantifying temporal and spatial variation of resistance to azoxystrobin and propiconazole. We consider fungicide resistance to be a stable, heritable trait that results in the reduction of the sensitivity of a fungus to a fungicide (McGrath 2004).

MATERIALS AND METHODS

Isolate acquisition. *Z. tritici* was surveyed to quantify resistance to azoxystrobin and propiconazole in commercial winter wheat fields in the Willamette Valley of Oregon (Figures 4.1 and 4.2) in June 2014, January 2015, May 2015, and January 2016. Nine to 17 fields were sampled at each sampling date. Fields were selected in 2014 based on presence of STB, physical accessibility, and grower cooperation. In subsequent field collections, the "next nearest" winter wheat fields to the fields sampled in 2014 were chosen. All fields sampled in this study were autumn-planted. While wheat is often grown in rotation with other crops in the Willamette Valley, three of the fields sampled in 2014 were re-planted to winter wheat the following year, allowing us the opportunity to sample these same fields in

June 2014, January 2015, and May 2015. These fields are indicated with open circles, squares, and triangles (Figures 4.1-4.4).

Each field was sampled by walking two 300m transects, separated by 300m. Five leaves with at least one *Z. tritici* lesion containing pycnidia were collected at 30 m intervals along each of the two transects, for 20 intervals total. For early season collections occurring in January 2015 and 2016, third or fourth leaves were collected (Large 1954). For late season collections occurring in June 2014 and May 2015 (approximately three weeks after final fungicide application), only flag leaves were collected. The location of transects within each field was determined by the size and shape of the field, leaving a minimum of 150 m from the field edge and each of the transect lines in most fields. In fields that were too small to accommodate a 150m distance between transects and the field edges, the transects were centered symmetrically in the field, while maintaining 300 m between the two transects. The overall survey design was hierarchical, with the Willamette Valley at the highest "5°" scale. Within the Willamette Valley, two sub-regions were sampled; the South Willamette Valley and the North Willamette Valley, at the "4°" scale. The northern and southern regions of the Willamette Valley were divided by Highway 20 as it crosses Interstate 5 at 44.629° N, -123.061° W (i.e. fields above 44.629° N were considered North sites, fields below 44.629° N were considered South sites). Fields within the two North and South regions were the "3°" scale, and transects within the field were the "2°" scale and sampled within the transect was the "1°" scale. In summary, the overall hierarchical scheme of the study is as follows: within Valley

(between North and South regions), within region (among fields), within field (between transects), and within transect (among samples).

The first five leaves with *Z. tritici* infections observed within a 1.5 m radius of each transect interval were collected, bagged, labeled, and transported to the laboratory. In the laboratory, the leaf with the most abundant *Z. tritici* infection of the five leaves from the interval was chosen, and mounted to a labeled sheet of paper with laboratory tape (VWR international, Radnor, PA) and pressed in a custom-made botanical press layered with newspaper. All leaves were pressed the same day they were collected from the field, and left to dry at bench top in the press for up to six weeks before processing. The four remaining leaves from each interval were put into long term storage as back up.

Spores were harvested from individual pycnidia by placing the preserved leaves overnight in Petri dishes with moistened filter paper. The next morning, one cirrhus containing extruded pycnidiospores was transferred with sterile forceps from the wheat leaf onto to a yeast malt agar (4 g of yeast extract, 4 g of malt extract, 4 g of sucrose, 15 g of agar, and 1 liter of water) Petri dish amended with gentamicin (10 ml/liter) to prevent bacterial growth. Each isolate evaluated in the study originated from one, mono-pycnidial cirrhus.

To generate the response variable, *Z. tritici* isolates (n=20 isolates per field) were tested from each transect interval for qualitative resistance to azoxystrobin and quantitative resistance to propiconazole. To test for resistance to azoxystrobin, each isolate was plated onto a control Petri dish without fungicide, and an amended petri dish with 1ppm technical grade azoxystrobin.. Growth of the isolate was visually

rated 3 days after plating. If the isolate on the amended plate had no growth compared to the control plate; it was scored as sensitive (0) (i.e. the *Z. tritici* isolate could not grow in the presence of the fungicide). If the isolate on the amended plate grew at the same rate as the control plate, it was scored as resistant (1) (i.e. the *Z. tritici* isolate was able to grow in the presence of the azoxystrobin fungicide) (Supplemental Figure 4.1).

To test for sensitivity to propiconazole, each isolate was plated onto a control Petri dish without fungicide, and an amended Petri dish with 0.1 ppm propiconazole. Growth of the isolate was visually rated five days after plating, and was scored on a quantitative scale of sensitive (0) – highly insensitive (5), using the control plate as a reference (Supplemental Figure 4.2).

Phenotyping validations. Isolates originating from the January 2015, May 2015, and January 2016 were stored on YMAa at 26°C for six months after phenotyping**.** Within six months of the original isolations, 6.8% of isolates were randomly chosen and blindly re-phenotyped for azoxystrobin resistance and propiconazole sensitivity.

STATISTICAL ANALYSIS

Temporal. In this study, the field is the replicate, and the 20 isolates evaluated from each field are considered sub-samples. For both propiconazole and azoxystrobin, collection time was treated as a discrete, non-continuous explanatory variable. Azoxystrobin data are quasibinomial, non-normally distributed with 20 data points each, “0” (fungicide-sensitive) and “1” (fungicide-resistant) scores used to generate a proportion of resistant isolates out of 20 total isolates per field.

All analyses were performed in R version 3.1.2 (2014-10-31). R Studio, Version 0.98.1091 RStudio, Inc (R Core Team 2013) was also used. We utilized the glm() function in R to construct a generalized linear model with a logit link to estimate the change in proportion of *Z. tritici* isolates resistant to azoxystrobinover the four collection times in this study. The quasibinomial was ultimately chosen over the binomial generalized liner model to account for observed dispersion in the variance of deviance residuals. Using the quasibinomial model, the estimable() function of the gmodels() package was used to estimate differences in the proportion of azoxystrobin-resistant isolates by collection time using a comparison vector.

Propiconazole sensitivity data met assumptions of normally distributed data; therefore, an analysis of variance was performed to evaluate collection time as a discrete fixed effect in the linear model. The lsmeans() function in the lsmeans package was utilized to obtain pairwise comparisons of propiconazole resistance by collection time.

In addition to the azoxystrobin resistance and propiconazole sensitivity as a function of collection time, several other covariates along with the field location data were evaluated. Covariates included wheat variety, calendar date in which the field was planted, and number of fungicide applications made to the field. We organized the fungicide applications into categories that included total fungicide applications, total strobilurin applications, total triazole applications, total azoxystrobin applications, and total propiconazole applications. We also generated a categorical response variable from the spray records and investigated whether or not any sprays at all (“1”) versus no sprays at all (“0”) affected fungicide resistance.

Spatial AMOVA. The poppr.amova() function in the R statistical software package 'poppr' was used to complete molecular analysis of variance (Kamvar, Tabima, and Grünwald 2014; Kamvar 2016). While the poppr package was designed for molecular data,poppr was utilized to analyze our phenotypic fungicide resistance data in this study. To accomplish this, the azoxystrobin resistance phenotype was treated as one locus with two possible alleles (qualitative scale: '0' for sensitive, '1' for resistant). The propiconazole sensitivity phenotype was treated as one locus with six possible alleles (quantitative scale: '0' for sensitive, '1', '2', '3', '4', '5' for different levels of insensitive).

Bartel's test. To determine if fungicide resistance phenotypes were randomly distributed or non-aggregated in the field, we utilized the Bartels.test() function in the lawstat() package in R to perform a two-sided Bartel's test for each surveyed field. Twelve fields scored azoxystrobin-resistant at every transect interval. Thus, these 100% resistant fields were omitted due to a lack of transect-level spatial variation, leaving 44 remaining fields to evaluate for randomness of the azoxystrobin phenotype with the Bartel's test. All 56 fields were eligible for the Bartel's test with respect to the propiconazole sensitivity phenotype. To complete the Bartel's test, both transects were assumed to be one "run" with 20 data points at each 30 m transect interval. Aggregation or randomness of azoxystrobin resistance and propiconazole sensitivity phenotypes were evaluated for each field independently. Given the limited sample size of 20 isolates per field, we elected to apply a conservative Bonferroni correction to the Bartel's test results.

RESULTS

The re-phenotyping confirmed the accuracy of our methodology for the qualitative azoxystrobin resistance ratings at 100% accuracy. Regression of the reevaluated and original propiconazole sensitivity scores showed a slope of 0.89 and a R^2 of 0.69. Overall, 67% of reevaluated isolates had a score identical to the original, 25% differed by one unit on the 0-5 scale, 6.5% differed by two units, 1.3% differed by three units, and no reevaluated isolate differed by more than three units

Temporal changes. There is strong evidence that the mean proportion of azoxystrobin-resistant *Z. tritici* increased over time ($F_{3,52} = 20.79$, $p<0.0001$) (Figures 4.1 and 4.3). The proportion of resistant *Z. tritici* isolates collected in January 2015 was greater than the proportion of resistant isolates collected in June 2014, and this difference is marginally significant ($t_{52}=1.86$, $p=0.0689$). The greatest increase in the proportion of resistant isolates occurred from January 2015 to May 2015, ($t_{52}=3.53$, $p=0.0009$). No change in azoxystrobin resistance was detected between the last two collection times of May 2015 to January 2016 ($t_{52}=0.64$, $p=0.5201$). The three fields sampled from the two-year wheat rotation follow the same general trend as the overall mean azoxystrobin resistance. In fact, the field identified with the open triangle shape (Figure 4.3), received no strobilurin fungicide applications for the entire duration of the study, and even so this field displays an increase in axozystrobin resistance (Figure 4.3).

There is strong evidence that the mean propiconazole sensitivity depended on collection time ($F_{3,52} = 8.738$, $p<0.0001$). This relationship with time is likely driven by the significant increase in sensitivity in January 2015 ($t_{52}=5.12$, $p<0.0001$), and the subsequent decrease in sensitivity in May 2015 ($t_{52}=-3.148$, $p=0.0141$) (Figure 4.2).

The three fields sampled from the two-year winter wheat rotation follow the same general trend as the overall mean propiconazole sensitivity.

Other than collection time, none of the covariates investigated affected fungicide resistance.

Spatial AOMVA. At all four collection times, for both azoxystrobin resistance and propiconazole sensitivity, the greatest component of covariance for the resistant/sensitive phenotype was at the finest, within-transect, "1°" hierarchical scale (Tables 4.1 and 4.2). For azoxystrobin resistance, the within transect-level variance averaged 93.8% across the four collection times (Table 4.1). For propiconazole sensitivity, the within transect level "1°" hierarchical scale averaged 87.7% variance across the four collection times (Table 4.2).

Spatial aggregation within transects. We failed to reject the null hypothesis and concluded that phenotypes were randomly distributed within transects for both azoxystrobin resistance and propiconazole sensitivity phenotype in all surveyed fields.

DISCUSSION

We identified different temporal dynamics for *Z. tritici* resistance to azoxystrobin (QoI) and propiconazole (DMI) fungicides in commercial winter wheat fields of the Willamette Valley, Oregon. Azoxystrobin resistance increased rapidly from 63% to nearly 93% resistance over the two-year study period. Our results are very similar to that found in Ireland, where a rapid trend of increasing strobilurin resistance was documented in commercial wheat fields from the 2004 to 2005 growing season (McCartney et al. 2007). The rapid increase of azoxystrobin

resistance measured in our study is not unexpected, owing to the qualitative effect of the G143A mutation. Because of the near-complete cross resistance among strobilurin fungicides (QoI Working Group 2016), we considered the number of total strobilurin applications per year to be an indication of selection for resistance. The mean number of strobilurin applications (either alone or in mixture with another fungicide) was 0.26 and 0.23 for the 2014 and 2015 seasons, respectively, which would provide some selection pressure for resistance.

Results are not completely explained by fungicide selection within fields, however, since azoxystrobin resistance increased over the winter months, a time when no fungicide applications were made to commercial fields. In addition, resistance increased over time even in fields that had no strobilurin applications over a two-year period. These observations suggest there may be adapted gene complexes that favor strobulurin resistance. It is also noteworthy to mention a large scale inoculated field trial to evaluate competition between resistant and sensitive isolates suggests when the resistant phenotype is at high frequency in the population, fitness of the resistant phenotype is neutral or favored (Hagerty et al., in prep).

The temporal pattern for *Z. tritici* propiconazole resistance was different from that of azoxystrobin resistance in commercial winter wheat fields of the Willamette Valley. There appears to be variation of propiconazole resistance among isolates sampled based on our ratings. Despite the apparent variation, propiconazole sensitivity was more stable than that of azoxystrobin. This difference in resistance is not unexpected, given the quantitative inheritance of triazole resistance (Cools and Fraaije 2013). In contrast to azoxystrobin, we found some evidence for an increase in

sensitivity during the winter months, when no fungicides were applied (Figure 4.2); this result suggests a possible fitness cost associated with mutations for propiconazole insensitivity. Given incomplete cross-resistance among the triazole fungicides (Leroux et al. 2007), we used number of applications of propiconazole as a measure of selection pressure. Mean number of propiconazole applications (either alone or in mixture with another fungicide) was 1.6 and 1.05 in 2014 and 2015, respectively.

Despite relative stability of propiconazole tolerance measured in this study, there is evidence for decreased sensitivity to propiconazole over time in the Willamette Valley. Evidence includes multiple mutations in CYP51 that were detected in 2012 *Z.tritici* populations that were not present in 1992 populations (Estep et al. 2015), decreased sensitivity of *Z.tritici* to propiconazole in response to field application of propiconazole (Hayes et al. 2015), and current field applications of propiconazole in the Willamette Valley which appear to be less effective than when the fungicide was first used to control STB in the late 1980s and early 1990s (Mundt C.C, unpublished).

Each mutation contributing to triazole sensitivity in CYP51 has a small effect (Mullins et al. 2011), thus imparting smaller selection pressure than the strobilurins. Evaluation of isolates collected from the Willamette Valley in 2012 indicated multiple CYP51 mutations relevant to triazole insensitivity. However, each isolate had only a single mutation for triazole insensitivity (Estep et al. 2015). We expect that, as in Europe (Dooley 2015), the next evolutionary step will be for an accumulation of multiple mutations for triazole insensitivity in individual *Z. tritici* genotypes.

Resistance evaluation methods were chosen for speed and low cost. We evaluated growth of *Z. tritici* on azoxystrobin-amended media, rather than with molecular analysis of the G143A mutation. Subsampling to validate our technique showed this assay to be 100% repeatable in this study. A previous study (Hagerty and Mundt 2016) showed complete correspondence between growth on 1.0 ppm azoxystrobin and presence of the G134A mutation. We developed an ordinal scale of *Z. tritici* on media amended with 0.1 ppm propiconazole as a measure of degree of resistance, which is an alternative to the expensive and time consuming process of determining EC50s on a range of concentrations. Though potentially subjective, we found this assay to be highly repeatable in subsequent retesting of randomly selected isolates.

The vast majority of the spatial variation for resistance to both fungicides was within-transect. Little variation was observed at greater hierarchical scales of the study including North Valley vs. South Valley within the Willamette Valley, among fields sampled within the region, or between transects within the field.

Variation for neutral markers will not always be associated with variation for adaptive traits under selection, such as fungicide resistance. For *Z. tritici,* correlation between neutral and phenotypic markers has been shown for some traits (Zhan et al. 2002) but not others (Mundt et al. 1999). Our results with the variation for fungicide resistance correlate very closely with the fine spatial scale detected with selectively neutral markers in several studies (Linde, Zhan, and McDonald 2002).

Sensitivity for both fungicides is fairly uniform in the Willamette Valley. This can be explained by a combination of long-distance dispersal of ascospores, coupled

with multiple mutation events for fungicide resistance (Fraaije et al. 2005; Torriani et al. 2009). Molecular analyses suggest several independent mutations for G143A as well as multiple different mutations influencing triazole sensitivity at the CYP51 locus (Estep et al. 2015; Torriani et al. 2009).

A very important question for understanding the biology and management of a plant pathogen is the degree to which selection is local (e.g. on farm), or regional (e.g. between farms). As discussed, we found temporal increase of azoxystrobin resistance in fields regardless of whether the fields were treated with a strobilurin fungicide or not. One interpretation of this result is that long-distance dispersal of ascospores among fields results in changes in fungicide sensitivity based on region-wide applications of fungicide resistance, coupled with a large amount of dispersal among fields. For example, heavy fungicide use by a particular farm in one region could have the capacity to drive fungicide resistance at neighboring farms within the region, given the wind-dispersed ascospore stage of *Z. tritici*.

In contrast, we also have evidence for local impacts. For example, a field that was formerly a orchardgrass seed field had the lowest azoxystrobin resistance in 2016, despite being located less than 2 km away from a field that had shown very high resistance in the two previous years. In addition, despite the near fixation of the azoxystrobin resistance phenotype region wide, azoxystrobin resistance at Oregon State University's Botany and Plant Pathology Field Laboratory was only 12% and 22% in 2014 and 2015, respectively (Hagerty et al., in prep). This University research farm is on the edge of a small city with relatively low surrounding commercial wheat acreage and very few wheat trials (and fungicide applications) relative to the

commercial production system in the region. This combined evidence suggests more local, within-field ascospore dispersal. Otherwise we would have expected to see much higher levels of resistance at the University research farm, and in the field coming out of the long-term orchardgrass rotation. It is possible that this dynamic is the result of both local and region wide fungicide resistance, simultaneously. The local and regional coupled dynamic is consistent with long distance dispersal, where a large portion of propagules remain local, but with fat-tails that can disperse long distances (Mundt et al. 2009).

Willamette Valley wheat growers have quickly adapted to reports of fungicide resistance that were disseminated by University researchers and Extension personnel. Adaptations include the discontinuation of the strobilurins for STB control in favor of multiple triazoles and SDHIs. A greater emphasis has also been placed on planting STB resistant winter wheat varieties, and the consideration of cultural controls with the STB disease cycle. Cultural controls specifically include delaying planting date as to avoid the early season ascospore showers (Mundt, research in progress).

As in Europe, fungicide resistance has become a substantial problem for control of STB in the Willamette Valley of Oregon. We found variation in sensitivity to representatives of two major fungicide classes. Though temporal changes in resistance to a fungicide with qualitative resistance was much more rapid than that of a fungicide with quantitative resistance, insensitivity to both fungicides was distributed randomly throughout the region. The vast majority of resistance variation was at the smallest spatial scale studied, which is consistent with prior studies of neutral markers. The influence of local versus regional selection for resistance is

unclear, yet important for development of resistant management strategies, and suggests a need to better understand the spatiotemporal dynamics of fungicide resistance at the landscape scale.

Table 4.1. Analysis of molecular variance for phenotypic azoxystrobin resistance of *Zymoseptoria tritici* to azoxystrobin fungicide data surveyed from commercial winter wheat fields in the Willamette Valley, Oregon.

	Collection 1				Collection 2			
Hierarchical level	Variation (%)	Φ	Mean sq.	df	Variation (%)	Φ	Mean sq.	df
Between North and South regions	-0.64	-0.006	0.008	1	-1.39	-0.014	0.105	1
Between fields within regions	-0.64	-0.006	0.235	13	1.00	0.010	0.131	7
Between transects within field	3.09	0.031	0.198	15	0.70	0.007	0.099	9
Within transect	98.19	0.018	0.185	270	99.69	0.003	0.056	161
	Collection 3				Collection 4			
Between North and South regions	-0.27	-0.003	0.105	1	-2.56	-0.026	0.000	1
Between fields within regions	2.67	0.027	0.131	15	13.77	0.134	0.227	13
Between transects within field	7.04	0.072	0.099	17	2.00	0.023	0.063	15
Within transect	90.56	0.094	0.056	300	86.79	0.132	0.051	270

Table 4.2 Analysis of molecular variance for phenotypic propiconazole fungicide sensitivity of *Zymoseptoria tritici*, data surveyed from commercial winter wheat fields in the Willamette Valley, Oregon.

	Collection 1				Collection 2			
Hierarchical level	Variation (%)	Φ	Mean sq.	df	Variation (%)	Φ	Mean sq.	df
Between North and South regions	-1.23	-0.012	1.562	1	1.76	0.018	1.948	1
Between fields within regions	12.35	0.122	2.839	13	1.46	0.015	0.976	7
Between transects within field	6.48	0.073	1.060	15	3.40	0.035	0.795	9
Within transect	82.40	0.176	0.593	270	93.39	0.066	0.584	161
	Collection 3				Collection 4			
Between North and South regions	-1.16	-0.012	1.148	1	-1.52	-0.015	0.553	1
Between fields within regions	12.28	0.121	2.583	15	9.47	0.093	1.829	13
Between transects within field	0.91	0.010	0.740	17	4.84	0.053	0.763	15
Within transect	87.98	0.120	0.672	300	87.20	0.128	0.491	270

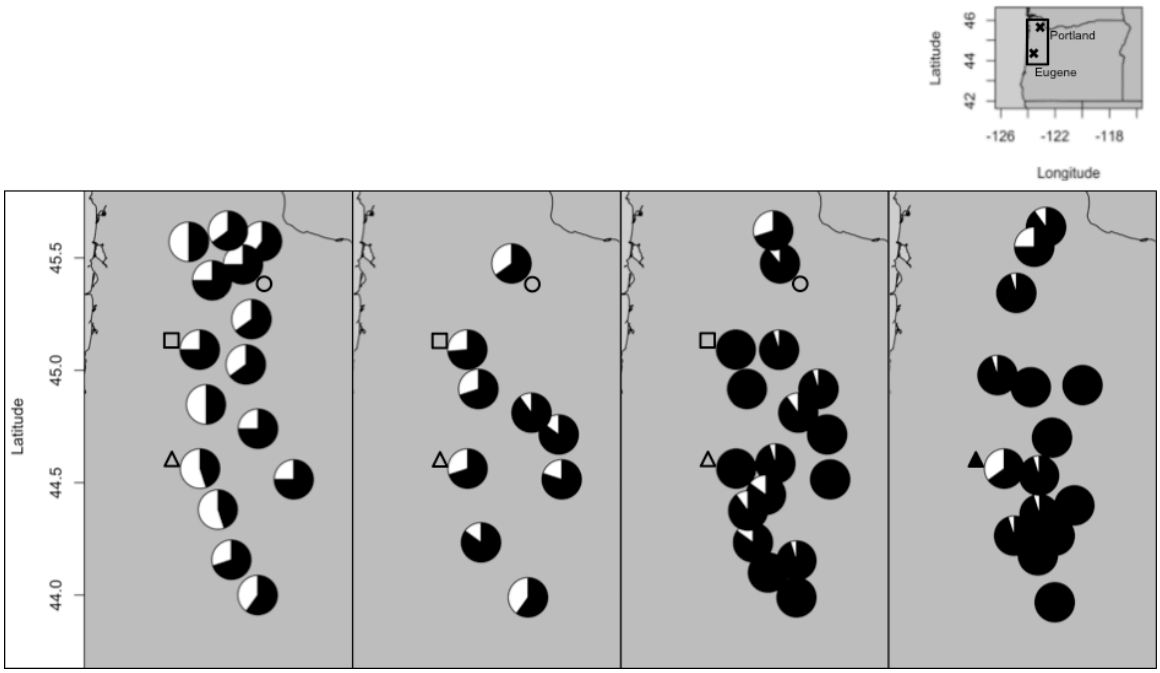


Figure 4.1. Inset map of Oregon in the upper right shows location of the study area, with the rectangle in the Northwest Oregon generally defining the Willamette Valley between Portland and Eugene. Geographical locations were sampled for *Zymoseptoria tritici* in field populations for resistance to azoxystrobin fungicide. Each circle represents one winter wheat field (n=20 isolates), the black portion: QoI resistant isolates, white portion: QoI sensitive isolates. The open circle, triangle, and square correspond to commercial fields planted in a two-year winter wheat rotation; closed triangle represents a field across the highway from the open triangle.

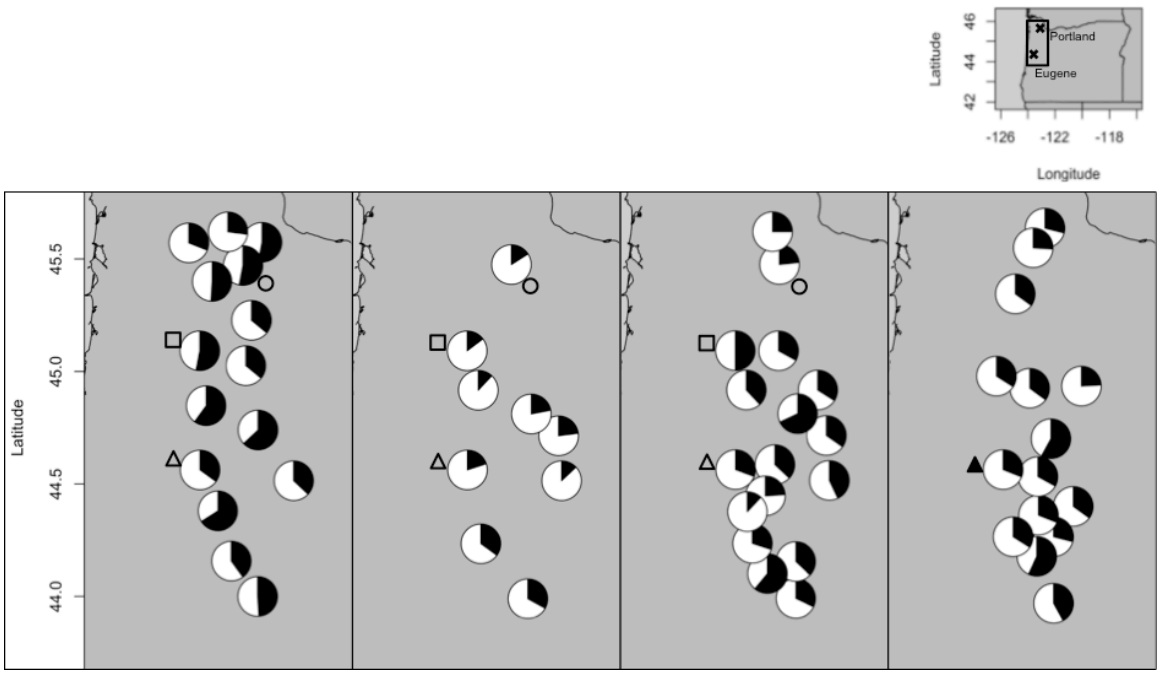


Figure 4.2. Inset map of Oregon in the upper right shows location of the study area, with the rectangle in the Northwest Oregon generally defining the Willamette Valley between Portland and Eugene. Geographical locations were sampled for *Zymoseptoria tritici* commercial field populations for resistance propiconazole fungicide. Each circle represents one commercial winter wheat field (n=20 isolates), the black portion: propiconazole sensitivity 0-5. The open circle, triangle, and square correspond to commercial fields planted in a two-year wheat rotation; closed triangle represents a field across the highway from the open triangle.

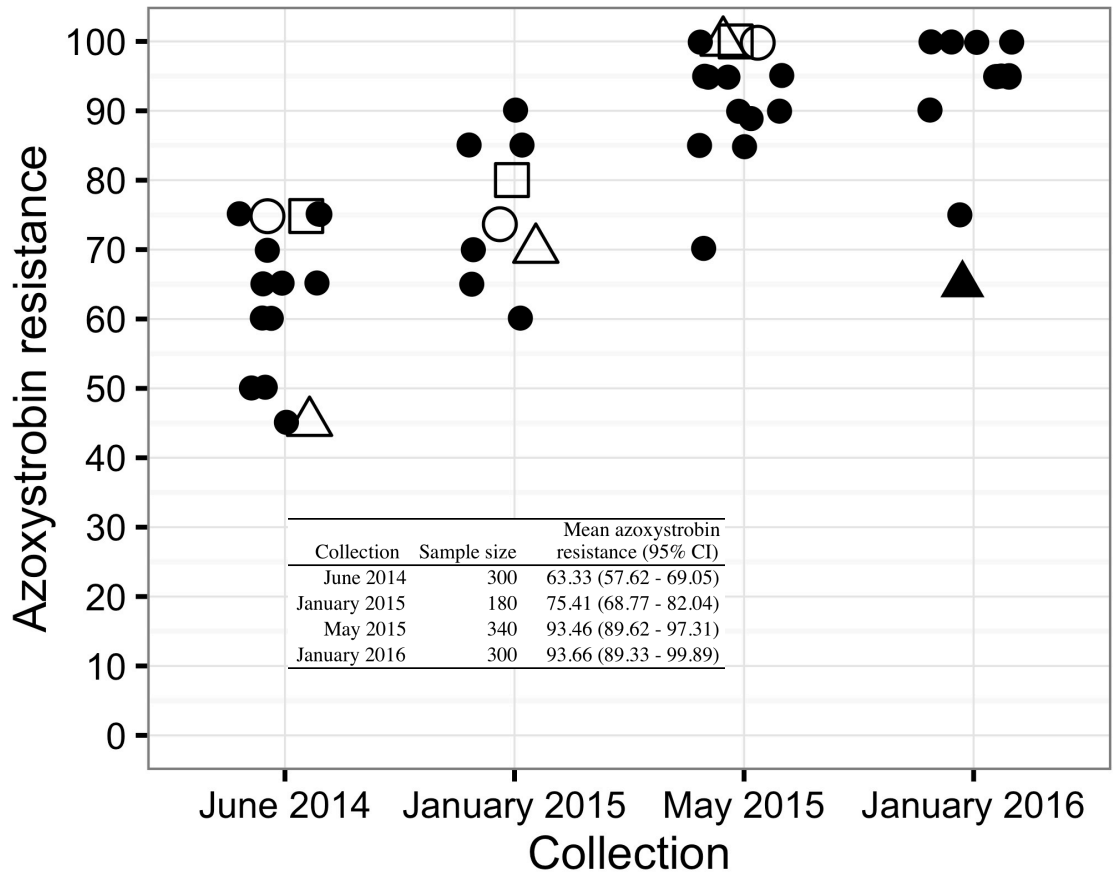


Figure 4.3. Each point represents the proportion of azoxystrobin-resistant *Zymoseptoria tritici* isolates sampled from a commercial winter wheat field of the Willamette Valley, Oregon at four seasonal collection times: June 2014, January 2015, May 2015, and January 2016 (n=20 isolates sampled per field). The open circle, square, and triangle represent three commercial fields planted in a two-year wheat rotation. The closed triangle in January 2016 represents a field directly across the highway from the field represented with the open triangle in May 2015. The table contains the sample size of isolates tested for azoxystrobin resistance, the mean resistance, and the 95% confidence interval for each collection of the survey. Points are plotted using geom_jitter() to aid visualization and avoid over-plotting within the collection time.

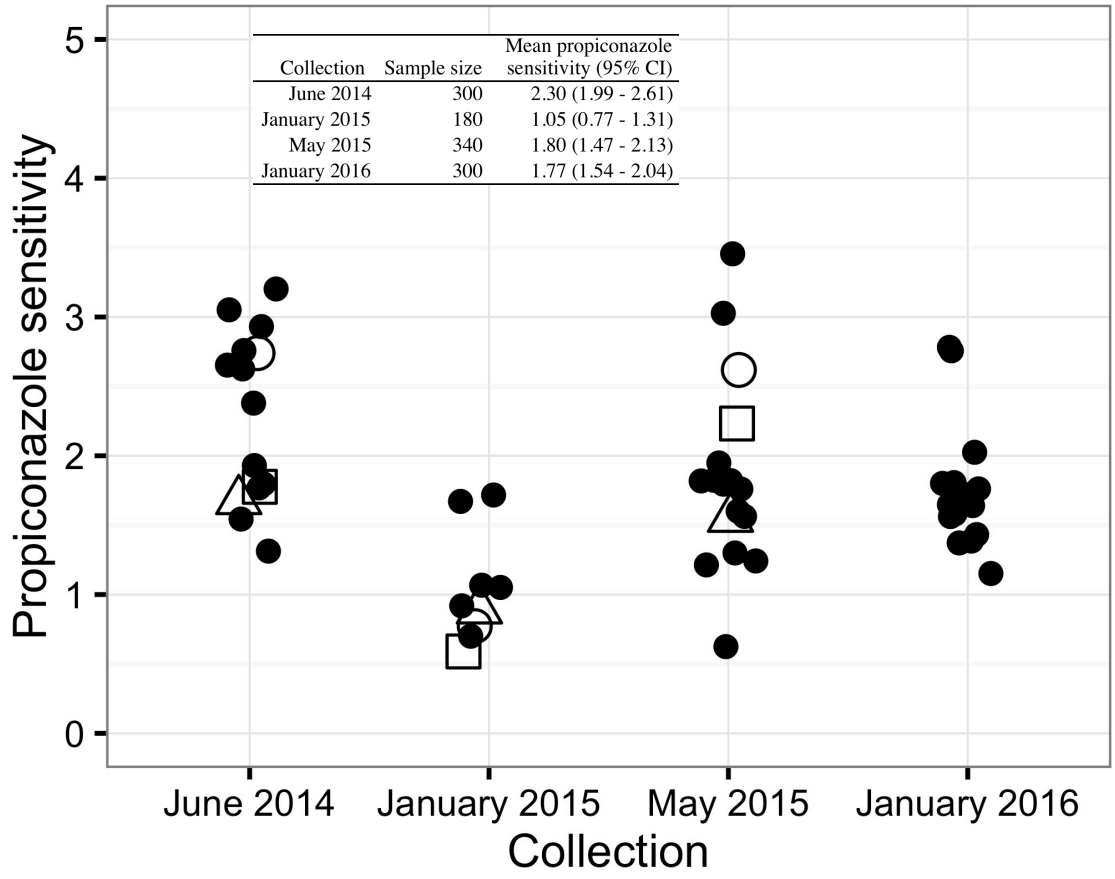
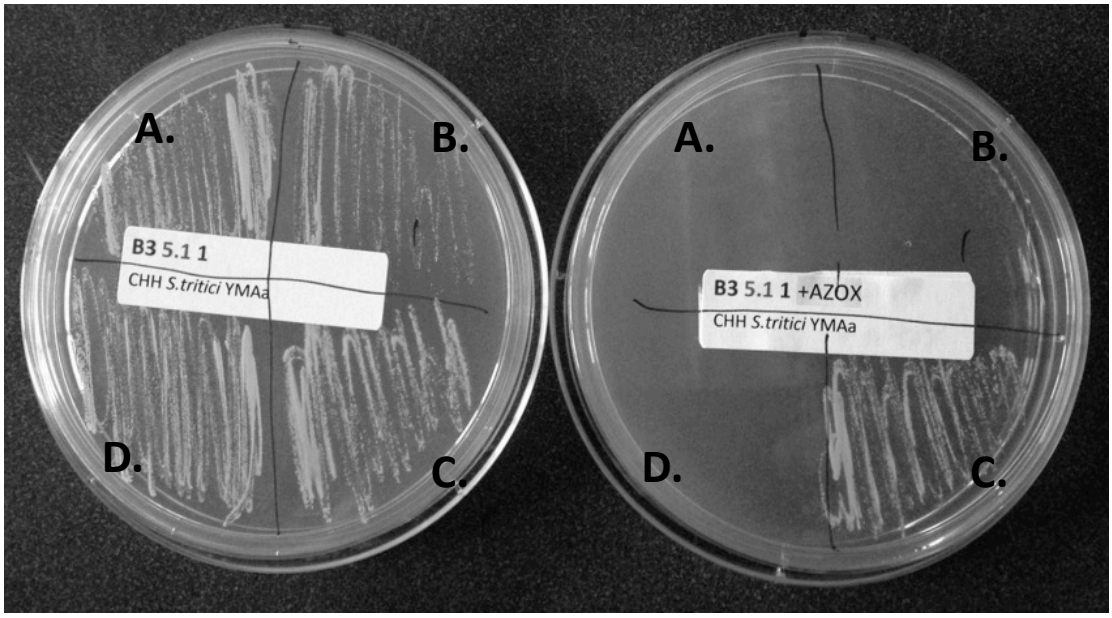
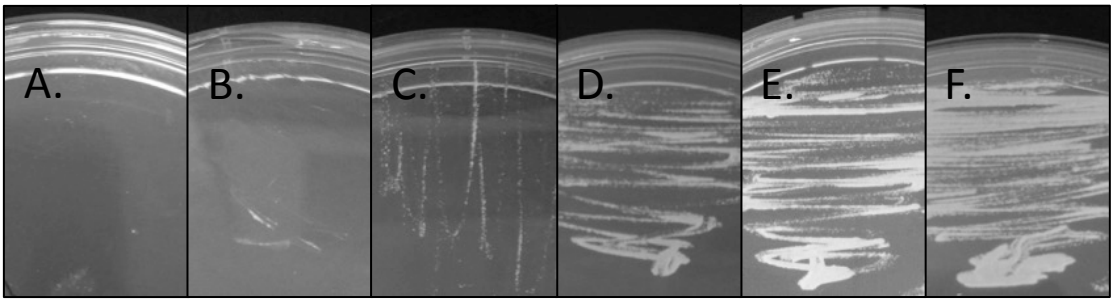


Figure 4.4. Each point represents the mean propiconazole sensitivity of *Zymoseptoria tritici* isolates sampled from a commercial winter wheat field of the Willamette Valley, Oregon at four seasonal collection times: June 2014, January 2015, May 2015, and January 2016 (n=20 isolates sampled per field). The circle, square, and triangle represent three commercial fields planted in a two-year wheat rotation. The table contains the sample size of isolates tested for propiconazole sensitivity, the mean sensitivity, and the 95% confidence interval for each collection of the survey. Points are plotted using geom_jitter() to aid visualization and avoid over-plotting within the collection time.



Supplemental Figure 4.1. Four *Zymoseptoria tritici* isolates collected from Willamette Valley field survey plated on yeast malt agar, right Petri dish un-amended control plate, left Petri dish amended with 1ppm azoxystrobin fungicide. Isolates A, B, and D azoxystrobin-sensitive, isolate C azoxystrobin-resistant.



Supplemental Figure 4.2. Six *Zymoseptoria tritici* isolates collected from Willamette Valley field survey plated on yeast malt agar amended with 0.1ppm propiconazole fungicide. Isolates display quantitative sale of sensitive (A) to insensitive (F).

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CHAPTER 5: CONCLUSION

Christina H. Hagerty

The three studies included in this research collectively provide a complex picture of the fungicide resistance dynamics of *Zymoseptoria tritici* in the Willamette Valley of Oregon, as well as that of fitness trade-offs typically associated with resistance mutations in general. Results from the greenhouse assay detailed in Chapter 2 demonstrate a putative fitness trade-off of fungicide-resistant *Z. tritici* in greenhouse assays. This result is consistent with central ecological and evolutionary theory that suggests fitness trade-offs may occur with resistance mutations in the absence of the selective agent. Results presented in Chapter 3 indicate that the magnitude and direction of fitness and competitive ability of fungicide-resistant *Z. tritici* is influenced by environmental variation. In addition, this study suggests that the fitness and competitive ability of fungicide-resistant *Z. tritici* may depend on the frequency of the resistance mutation in the population, with fitness being neutral or positive at high frequency. Lastly, in Chapter 4, results of the hierarchical survey of propiconazole and azoxystrobin-resistant *Z. tritici* indicated very different temporal dynamics for resistance to each of the chemicals monitored. A rapid increase in resistance to azoxystrobin was measured, and the increase in resistance was independent of fungicide use. In contrast, propiconazole resistance stayed relatively stable over time. We conducted analyses to understand more about the amount of variation present at each hierarchical scale of the survey, and found that the great majority of variation was at the smallest scale of the study. We also conducted analyses to explore the pattern and potential aggregation of the fungicide-resistant

phenotype within commercial wheat fields of the Willamette Valley. We found the fungicide resistance phenotype was randomly distributed within each of the fields sampled.

The combined effort of these studies demonstrates that the dynamics of fungicide resistance can be variable and somewhat stochastic. Results of these studies have important implications for predictive modeling of fungicide resistance emergence and spread in an agricultural landscape. Beyond implications for fungicide resistance in agriculture, results may have relevance antimicrobial and pesticide resistance in other human, plant, and insect systems.

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