Temporal Dynamics and Spatial Variation of Azoxystrobin and Propiconazole Resistance in *Zymoseptoria tritici*: A Hierarchical Survey of Commercial Winter Wheat Fields in the Willamette Valley, Oregon

Christina H. Hagerty, Nicole P. Anderson, and Christopher C. Mundt

First author: Department of Botany and Plant Pathology, Columbia Basin Agricultural Research Center, 48037 Tubbs Ranch Road, Adams, OR 97801; second author: Department of Crop and Soil Science, Oregon State University, 2050 NE Lafayette Ave., McMinnville 97128; and third author: Department of Botany and Plant Pathology, Oregon State University, 2082 Cordley Hall, 2701 SW Campus Way, Corvallis 97331. Accepted for publication 31 October 2016.

ABSTRACT

Fungicide resistance can cause disease control failure in agricultural systems, and is particularly concerning with *Zymoseptoria tritici*, the causal agent of Septoria tritici blotch of wheat. In North America, the first quinone outside inhibitor resistance in *Z. tritici* was discovered in the Willamette Valley of Oregon in 2012, which prompted this hierarchical survey of commercial winter wheat fields to monitor azoxystrobin- and propiconazole-resistant *Z. tritici*. 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, frequency of azoxystrobin-

resistant isolates increased from 63 to 93% from June 2014 to January 2016. Resistance to azoxystrobin increased over time even within fields receiving no strobilurin applications. Propiconazole sensitivity varied over the course of the study but, overall, did not significantly change. Sensitivity to 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 (within the transect) 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.

Pathogen populations with greater genetic diversity and sexual recombination typically have a greater risk for developing fungicide resistance (Linde et al. 2002). Regional surveys for fungicide resistance in the highly variable fungus *Zymoseptoria tritici* have revealed that many fungicides commonly used to control Septoria tritici blotch (STB) epidemics are no longer effective due to the buildup of resistance in local *Z. tritici* populations (Drabešová et al. 2013; Estep et al. 2015; Siah et al. 2014). In Europe, strobilurins (quinone outside inhibitors [QoI]) are no longer used for STB control due to near-complete resistance. Strobilurin resistance in *Z. tritici* is usually conferred by a single amino acid mutation in the mitochondrial cytochrome *b* gene where, at codon 143, glycine is substituted by alanine (G143A) (Fraaije et al. 2005). This substitution leads to qualitative or near-complete resistance. Remaining STB management options in Europe include the triazoles and the succinate dehydrogenase inhibitors (SDHI) (Dooley 2015). Triazole fungicides act as demethylation inhibitors (DMI) and interfere with sterol biosynthesis in the fungus (Thompson 1975). Twenty-four residues within the *CYP51* gene have been identified as subject to mutation (Cools and Fraaije 2013). Furthermore, the residues act in concert with at least 49 possible combinations of mutations identified in the *CYP51* gene, giving rise to quantitative resistance (Dooley 2015). Similar to the strobilurins, resistance to SDHI in *Z. tritici* is qualitative, with a single amino-acid substitution conferring resistance (Skinner et al. 1998). SDHI 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, prompting the release of propiconazole (Syngenta Crop Protection LLC) 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 (C. C. Mundt, unpublished). The first strobilurin fungicide was registered for wheat in Oregon in 2000 (Pscheidt and Ocamb 2000). Blended products combining a triazole and a strobilurin were recommended for STB management in 2004 (Pscheidt and Ocamb 2005). In the mid-2000s, new, aggressive, temperature-tolerant races of stripe rust (*Puccinia striiformis*) (Hovmøller et al. 2008) arrived in the Willamette Valley that were not sufficiently controlled by high temperature and 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 US\$7.50/ha when tank-mixed with herbicides), three to four fungicide applications per year have become standard when wheat prices are high (above US\$200/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; 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

Corresponding author: C. H. Hagerty; E-mail address: hagertyc@oregonstate.edu

*The e-Xtra logo stands for “electronic extra” and indicates that one set of supplementary data and two supplementary figures are published online.

(Fraaije et al. 2005), and resistance was then detected the following year in Ireland, France, Germany, and Denmark (Gisi et al. 2005). Gisi et al. (2005) monitored for resistance to strobilurins and triazoles from 1998 to 2004. No QoI-resistant isolates were detected until 2001 but, in 2003, resistance was detected simultaneously in Ireland, the 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 of four sites in Morocco. At the fourth site, however, they documented an increase from 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, after seasonal azoxystrobin fungicide applications, 87% of isolates sampled were resistant to azoxystrobin.

In contrast to strobilurins, triazoles are more stable and, generally, a lower frequency of *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 et al. 2005). Gradual shifts in triazole sensitivity have yet to result in complete loss of efficacy, and no field resistance has been reported (Cools et al. 2005; FRAC SBI Working Group 2015; Mavroeidi and Shaw 2006). However, resistance in *Z. tritici* isolates has been detected to all members of the triazole class (Cools et al. 2011). Because strobilurins are now ineffective at controlling STB in Europe, the continued heavy reliance on triazoles provides 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, for the first time, reported the development of strobilurin resistance in *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. 2016) were obtained and results indicated selection for a small reduction in sensitivity 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 that 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 et al. 1998). The spatial pattern of fungicide resistance in *Z. tritici* has yet to be 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 (Figs. 1 and 2) in June 2014, January 2015, May 2015, and January 2016. In all, 9 to 17 fields were sampled at each sampling date. Fields were selected in 2014 based on the presence of STB, physical accessibility, and grower cooperation. In subsequent field collections, the “next nearest” available winter wheat fields to the fields sampled in 2014 were chosen. All fields sampled in this study were autumn planted. Although wheat is often grown in rotation with other crops in the Willamette Valley, three of the fields sampled in 2014 were replanted to winter wheat the following year, allowing us the opportunity to sample these same fields in June 2014, January 2015, and May 2015. These three fields are indicated with open circles, squares, and triangles (Figs. 1 to 4). A closed triangle indicates a field sampled in January 2016 that is less than 2 km from a field sampled in previous years.

Each field was sampled by walking two 300-m transects, separated by 300 m. Five leaves each 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 3 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 between the field edge and each of the transect lines in most fields. In fields that were too small to accommodate 150 m 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 (Skinner et al. 1989). Within the Willamette Valley, two subregions 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 and fields below 44.629° N were considered South sites). Fields within the two North and South regions were the 3° scale, transects within the field were the 2° scale, and samples within the transect were 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). To generate the response variable, *Z. tritici* isolates ($n = 20$ isolates/field) were tested from each transect interval for qualitative resistance to azoxystrobin and quantitative resistance to propiconazole.

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, mounted to a labeled sheet of paper with laboratory tape (VWR international), 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 6 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 cirrus containing exuded pycnidiospores was transferred with sterile forceps from the wheat leaf onto to a yeast malt agar (YMA) (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 monopycnidial cirrus.

Fungicide resistance tests. To test for resistance to azoxystrobin, four isolates were plated onto a control petri dish split into four quadrants without fungicide, and the same four isolates were plated into each quadrant of an amended petri dish with technical-grade azoxystrobin at 1 µg/ml (Estep et al. 2015). Plating occurred by smearing one monopycnidial colony in an even pattern across one quadrant of the plate with a sterile bacteriological loop. Growth of the isolate was visually rated 3 days after plating. If the isolate on the amended plate had no growth compared with 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) (Supplementary Fig. S1). The qualitative measurement of resistance was based on degree of inhibition at a single dose.

To test for sensitivity to propiconazole, four isolates were plated onto each quadrant of a control petri dish without fungicide, and the same four isolates were plated in each quadrant of an amended petri dish with propiconazole at 0.1 µg/ml. Growth of the isolate was visually rated 5 days after plating, and was scored on a quantitative scale of sensitive (0) to highly insensitive (5), using the control plate as a reference (Supplementary Fig. S2).

We utilized one replicate per isolate for both azoxystrobin and propiconazole assays because the methodologies were established as highly repeatable (see “Phenotyping validations” below).

Phenotyping validations. Isolates collected in June 2014 were discarded after data collection, because this study had not yet been established as a longitudinal survey. However, isolates originating from January 2015, May 2015, and January 2016 were stored on

YMA at 22°C for 6 months after phenotyping. Within 6 months of the original isolations, 25 isolates of each of the three sampling times were randomly chosen and blindly rephenotyped for azoxystrobin resistance and propiconazole sensitivity.

Statistical analysis. Temporal analysis. In this analysis, the field was the replicate and the 20 isolates evaluated from each field were considered subsamples. For both propiconazole and azoxystrobin, collection time was treated as a noncontinuous explanatory variable. Azoxystrobin data were quasibinomial, nonnormally distributed with 20 data points each, and scores of 0 (fungicide sensitive) and 1 (fungicide resistant) were used to generate a proportion of resistant isolates out of 20 total isolates/field.

All analyses were performed in R version 3.1.2 (2014-10-31). R Studio, version 0.98.1091 RStudio, Inc. 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 azoxystrobin over the four collection times in this study. The quasibinomial was ultimately chosen over the binomial generalized linear 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 sensitivity by collection time.

In addition to azoxystrobin resistance and propiconazole sensitivity as a function of collection time, several other covariates

Fig. 1. Maps display proportion of azoxystrobin fungicide-resistant isolates sampled from commercial winter wheat fields in the Willamette Valley of Oregon over four sampling dates. Inset map of Oregon in the upper right shows location of the study area, with the rectangle in northwest Oregon generally defining the Willamette Valley between Portland and Eugene. Each of the four panels represents a sampling date of the study. 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 indicates quinone outside inhibitor (QoI)-resistant isolates and the white portion indicates QoI sensitive isolates. The open circle, triangle, and square correspond to commercial fields planted in a 2-year winter wheat rotation, and the closed triangle represents a field across the highway from the open triangle.

along with the field location data were evaluated. Other covariates were wheat variety, calendar date on 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 analysis of molecular variance. The `poppr.amova()` function in the R statistical software package `poppr` was used to complete analysis of molecular variance (AMOVA) (Kamvar et al. 2014). Although the `poppr` package was designed for molecular data, it 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 and 1 for resistant). The propiconazole sensitivity phenotype was treated as one locus with six possible alleles (quantitative scale: 0 for sensitive and 1, 2, 3, 4, and 5 for different levels of insensitive). Although the AMOVA procedure was initially implemented for DNA haplotypes, it applies to any marker system (Grunwald et al. 2016).

Bartel’s test. To determine whether fungicide resistance phenotypes were randomly distributed or nonaggregated 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 (Gastwirth et al. 2015). Twelve fields scored as azoxystrobin resistant at every transect interval. Thus, these 100% resistant fields were omitted because, by definition, no variation was present. This left 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, one 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/field, we elected to apply a conservative Bonferroni correction to the Bartel’s test results.

RESULTS

Temporal changes. The mean frequency of azoxystrobin-resistant *Z. tritici* increased over time from 63.33% of isolates collected at the beginning of the study to 93.66% of isolates collected at the end of the study ($F_{3,52} = 20.79$, $P < 0.0001$) (Figs. 1 and 3). The greatest increase in the proportion of resistant isolates occurred from 75.41% in January 2015 to 93.46% in May 2015, ($t_{52} = 3.53$, $P = 0.0009$). No change in azoxystrobin resistance was detected between the last two collection times from 93.46% resistant isolates in May 2015 to 93.66% resistant isolates in January 2016 ($t_{52} = 0.64$, $P = 0.5201$). The three fields sampled from the 2-year wheat rotation followed the same general trend as the overall mean frequency azoxystrobin resistance. An increase in azoxystrobin-resistant isolates was also observed in the field that received no strobilurin fungicide applications during the entire duration of the study (Fig. 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 was likely driven by the significant increase in sensitivity in January 2015 from a mean sensitivity of

Fig. 2. Maps display proportion of propiconazole fungicide-resistant isolates sampled from commercial winter wheat fields in the Willamette Valley of Oregon over four sampling dates. Inset map of Oregon in the upper right shows location of the study area, with the rectangle in northwest Oregon generally defining the Willamette Valley between Portland and Eugene. Geographical locations were sampled for *Zymoseptoria tritici* in commercial field populations for resistance to propiconazole fungicide. Each circle represents one commercial winter wheat field ($n = 20$ isolates): the black portion represents the average propiconazole tolerance score from 20 isolates collected per field on a qualitative 0-to-5 scale. The open circle, triangle, and square correspond to commercial fields planted in a 2-year wheat rotation, and the closed triangle represents a field across the highway from the open triangle.

1.05 ($t_{52} = 5.12$, $P < 0.0001$), and the subsequent decrease in sensitivity in May 2015 to a mean sensitivity of 1.80 ($t_{52} = -3.148$, $P = 0.0141$) (Fig. 2). The three fields sampled from the 2-year winter wheat rotation followed the same general trend as the overall mean propiconazole sensitivity.

Other than collection time, no significant differences in fungicide resistance frequency were observed among the other covariates, including wheat variety, calendar date on which the field was planted, and number of fungicide applications made to the field.

Spatial AMOVA. At all four collection times, for both azoxystrobin resistance and propiconazole sensitivity, the greatest component of covariance for the resistant or sensitive phenotype was at the finest, within-transect, 1° hierarchical scale (Tables 1 and 2). For azoxystrobin resistance, the within-transect-level variance averaged 93.8% across the four collection times (Table 1). For propiconazole sensitivity, the within-transect-level 1° hierarchical scale averaged 87.7% variance across the four collection times (Table 2). The Φ population differentiation statistics, used to test hypotheses about population differentiation, are also presented in Tables 1 and 2, and a higher Φ statistic generally represents a higher amount of differentiation (Grunwald et al. 2016). The Φ population differentiation statistics indicated that a higher population differentiation occurred at the within transect level.

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 phenotypes in all surveyed fields.

Phenotyping repeatability. The rephenotyping 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 an 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-to-5 scale, 6.5% differed by two units, 1.3% differed by three units, and no reevaluated isolate differed by more than three units on the six-point scale.

Fig. 3. Each point represents the proportion of azoxystrobin-resistant *Zymoseptoria tritici* isolates sampled from a commercial winter wheat field of the Willamette Valley of 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 2-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 overplotting within the collection time.

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 in Oregon. Frequency of azoxystrobin resistance increased rapidly from 63 to nearly 93% over the 2-year study period. Our results are very similar to that found in Ireland 10 years prior, where a rapid trend of increasing strobilurin resistance was documented in commercial wheat fields from the 2004–05 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, because 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 2-year period. These observations suggest that there may be adapted gene complexes that favor strobilurin resistance because the proportion of resistant isolates sampled increased in the absence of seasonal fungicide sprays. It is also noteworthy to mention that a large-scale inoculated field trial to evaluate competition between resistant and sensitive isolates suggests that, when the resistant phenotype is at high frequency in the population, fitness of the resistant phenotype is neutral or favored (C. H. Hagerty, R. C. Graebner, K. E. Sackett, and C. C. Mundt, unpublished).

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

Fig. 4. Each point represents the mean propiconazole sensitivity of *Zymoseptoria tritici* isolates sampled from a commercial winter wheat field of the Willamette Valley of 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 2-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 overplotting within the collection time.

2013). In contrast to azoxystrobin, we found some evidence for an increase in sensitivity during the winter months, when no fungicides were applied (Fig. 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 the 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 longer time periods 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. 2016), 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 (C. C. Mundt, unpublished).

Each mutation contributing to triazole insensitivity in *CYP51* has a small effect (Mullins et al. 2011) and, thus, is less affected by 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 azoxystrobin at 1.0 ppm and presence of the G143A mutation. We developed an ordinal scale of *Z. tritici* on media amended with propiconazole at 0.1 ppm as a measure of degree of resistance, which is an alternative to the expensive and time-consuming process of determining 50% effective concentration values on a range of concentrations. Though potentially subjective, we found this assay to be highly repeatable in subsequent retesting of randomly selected isolates. The sampling technique (to only choose leaves with pycnidia) may have biased results toward fungicide resistance in posttreatment samples; however, this technique was

necessary to complete this isolation-based study. Wider concentration ranges may be needed in future as more mutations potentially accumulate and sensitivity further declines.

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). We found that 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 versus South Valley within the Willamette Valley, among fields sampled within the region, or between transects within the field.

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. 2013; Torriani et al. 2009).

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 (Linde et al. 2002). 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, 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* and in the absence of strong fitness penalties.

In contrast, we also have evidence for local effects of fungicide resistance. For example, a field that was formerly an orchardgrass seed field had the lowest azoxystrobin resistance in 2016, despite being located less than 2 km from a field that had shown very high resistance in the two previous years. This local effect could potentially be explained by the splashed-dispersed asexual conidia driving local fungicide resistance effects.

In addition, despite the near fixation of the azoxystrobin resistance phenotype region wide, azoxystrobin resistance at Oregon State

TABLE 1. Analysis of molecular variance for phenotypes of azoxystrobin resistance in *Zymoseptoria tritici* isolates collected from commercial winter wheat fields in the Willamette Valley, OR^a

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

^a Var = variation, Φ = differentiation statistics for population differentiation, and MS = mean squares.

TABLE 2. Analysis of molecular variance for phenotypes of propiconazole sensitivity in *Zymoseptoria tritici* isolates collected from commercial winter wheat fields in the Willamette Valley, OR^a

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

^a Var = variation, Φ = differentiation statistics for population differentiation, and MS = mean squares.

University's Botany and Plant Pathology Field Laboratory was only 12 and 22% in 2014 and 2015, respectively (C. H. Hagerty, R. C. Graebner, K. E. Sackett, and C. C. Mundt, unpublished). 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 and conidia 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 SDHI. 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 the planting date so as to avoid the early-season ascospore showers (C. C. Mundt, unpublished).

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 relatively evenly 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.

ACKNOWLEDGMENTS

We thank K. E. Sackett, V. Le, and E. M. Sykes for technical help with managing isolates; R. C. Graebner for general support; Z. N. Kamvar for consultation with the poppr package (Tables 1 and 2); and P. I. Bennett for lending "mapprots" R code (Figures 1 and 2). This research was made possible due to the gracious support and land access granted by Willamette Valley wheat growers, and cooperation with local agricultural industry personnel. Field access was coordinated through generous efforts by T. Miller (Fitzmaurice Fertilizer Inc.) and D. McCready (Wilco-Winfield LLC).

LITERATURE CITED

- Chen, X. 2013. Review article: High-temperature adult-plant resistance, key for sustainable control of stripe rust. *Am. J. Plant Sci.* 4:608-627.
- Cools, H. J., and Fraaije, B. A. 2013. Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest Manage. Sci.* 69:150-155.
- Cools, H. J., Fraaije, B. A., Lucas, J. A., Dehne, H. W., Gisi, U., Kuck, K. H., Russell, P. E., and Lyr, H. 2005. Molecular examination of Septoria tritici isolates with reduced sensitivities to triazoles. Pages 103-114 in: *Modern Fungicides and Antifungal Compounds IV: 14th Int. Reinhardsbrunn Symp.* Friedrichroda, Thuringia, Germany. British Crop Protection Council.
- Cools, H. J., Mullins, J. G., Fraaije, B. A., Parker, J. E., Kelly, D. E., Lucas, J. A., and Kelly, S. L. 2011. Impact of recently emerged sterol 14 α -demethylase (*CYP51*) variants of *Mycosphaerella graminicola* on azole fungicide sensitivity. *Appl. Environ. Microbiol.* 77:3830-3837.
- Dooley, H. 2015. Fungicide-resistance management tactics: Impacts on *Zymoseptoria tritici* populations. Ph.D. thesis, University of Reading, Berkshire, United Kingdom. <http://centaur.reading.ac.uk/49314/>
- Drabesová, J., Ryšánek, P., Brunner, P., McDonald, B. A., and Croll, D. 2013. Population genetic structure of *Mycosphaerella graminicola* and quinone outside inhibitor (QoI) resistance in the Czech Republic. *Eur. J. Plant Pathol.* 135:211-224.
- Elmer, P. A. G., Gaunt, R. E., and Frampton, C. M. 1998. Spatial and temporal characteristics of dicarboximide-resistant strains of *Monilinia fructicola* and brown rot incidence in stone fruit. *Plant Pathol.* 47:530-536.
- Estep, L. K., Torriani, S. F. F., Zala, M., Anderson, N. P., Flowers, M. D., McDonald, B. A., Mundt, C. C., and Brunner, P. C. 2015. Emergence and early evolution of fungicide resistance in North American populations of *Zymoseptoria tritici*. *Plant Pathol.* 64:961-971.
- Estep, L. K., Zala, M., Anderson, N. P., Sackett, K. E., Flowers, M., McDonald, B. A., and Mundt, C. C. 2013. First report of resistance to QoI fungicides in North American populations of *Zymoseptoria tritici*, causal agent of Septoria tritici blotch of wheat. *Plant Dis.* 97:1511.
- Fraaije, B. A., Cools, H. J., Fountaine, J., Lovell, D. J., Motteram, J., West, J. S., and Lucas, J. A. 2005. Role of ascospores in further spread of QoI-resistant cytochrome *b* alleles (G143A) in field populations of *Mycosphaerella graminicola*. *Phytopathology* 95:933-941.
- FRAC SBI Working Group. 2015. Protocol of the discussions and recommendations of the SBI working group of the Fungicide Resistance Action Committee (FRAC). Minutes of the 2015 SBI Meeting, Recommendations for 2016, Frankfurt, Germany. Online publication. <http://www.frac.info/docs/default-source/sbi-wg/sbi-wg-archive/minutes-of-the-2015-meeting-recommendations-for-2016.pdf?sfvrsn=2>
- FRAC SDHI Working Group. 2013. Protocol of the discussions and use recommendations of the SDHI Working Group of the Fungicide Resistance Action Committee (FRAC). Minutes of the 2013 SDHI Meeting, Recommendations for 2014, Frankfurt, Germany. Online publication. <http://www.frac.info/docs/default-source/sdhi-wg/sdhi-meeting-minutes/minutes-of-the-2013-sdhi-meeting-recommendations-for-2014.pdf?sfvrsn=2>
- Gastwirth, J. L., Gel, Y. R., Wallace Hui, W. L., Lyubchich, Y., Miao, W., and Noguchi, K. 2015. lawstat: Tools for Biostatistics, Public Policy, and Law. R package version 1.14.4. Online publication. <https://cran.r-project.org/web/packages/lawstat/index.html>
- Gisi, U., Pavic, L., Stanger, C., Hugelshofer, U., Sierotzki, H., Dehne, H. W., Kuck, K. H., Russell, P. E., and Lyr, H. 2005. Dynamics of *Mycosphaerella graminicola* populations in response to selection by different fungicides. Pages 89-101 in: *Modern Fungicides and Antifungal Compounds IV: 14th Int. Reinhardsbrunn Symp.* Friedrichroda, Thuringia, Germany. British Crop Protection Council.
- Grunwald, N. J., Kamvar, Z. N., and Everhart, S. E. 2016. AMOVA. In: *Population Genetics in R*. Online publication. http://grunwaldlab.github.io/Population_Genetics_in_R/AMOVA.html
- Hagerty, C. H., and Mundt, C. C. 2016. Reduced virulence of azoxystrobin-resistant *Zymoseptoria tritici* populations in greenhouse assays. *Phytopathology* 106:884-889.
- Hayes, L. E., Sackett, K. A., Anderson, N. P., Flowers, M. D., and Mundt, C. C. 2016. Evidence of selection for fungicide resistance in *Zymoseptoria tritici* populations on wheat in Western Oregon. *Plant Dis.* 100:483-489.
- Hovmöller, M. S., Yahyaoui, A. H., Milus, E. A., and Justesen, A. F. 2008. Rapid global spread of two aggressive strains of a wheat rust fungus. *Mol. Ecol.* 17:3818-3826.
- Kamvar, Z. N., Tabima, J. F., and Grünwald, N. J. 2014. *Poppr*: An R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* 2:e281.
- Koepsell, P. A., Pscheidt, J. W., Forster, R., Mohan, S. K., Maloy, O., and Byther, R. 1989. *Pacific Northwest Plant Disease Control Handbook*. A Pacific Northwest Extension Publication: Oregon State University, Washington State University, and the University of Idaho, Corvallis, Oregon.
- Large, E. C. 1954. Growth stages in cereals illustration of the Feeke's Scale. *Plant Pathol.* 3:128-129.
- Leroux, P., Albertini, C., Gautier, A., Gredt, M., and Walker, A.-S. 2007. Mutations in the *CYP51* gene correlated with changes in sensitivity to sterol 14 α -demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Manage. Sci.* 63:688-698.
- Linde, C. C., Zhan, J., and McDonald, B. A. 2002. Population structure of *Mycosphaerella graminicola*: From lesions to continents. *Phytopathology* 92:946-955.
- Mavroeidi, V. I., and Shaw, M. W. 2006. Effects of fungicide dose and mixtures on selection for triazole resistance in *Mycosphaerella graminicola* under field conditions. *Plant Pathol.* 55:715-725.
- McCartney, C., Mercer, P. C., Cooke, L. R., and Fraaije, B. A. 2007. Effects of a strobilurin-based spray programme on disease control, green leaf area, yield and development of fungicide-resistance in *Mycosphaerella graminicola* in Northern Ireland. *Crop Prot.* 26:1272-1280.
- McDonald, B. A., and Mundt, C. C. 2016. How knowledge of pathogen population biology informs management of *Septoria tritici* blotch. *Phytopathology* 106:948-955.
- McGrath, M. T. 2004. What are fungicides? Online publication. *Plant Health Instruct.* doi:10.1094/PHI-I-2004-0825-01

- Mullins, J. G. L., Parker, J. E., Cools, H. J., Togawa, R. C., Lucas, J. A., Fraaije, B. A., Kelly, D. E., and Kelly, S. L. 2011. Molecular modelling of the emergence of azole resistance in *Mycosphaerella graminicola*. PLoS One 6:e20973.
- Mundt, C. C., Hoffer, M. E., Ahmed, H. U., Coakley, S. M., DiLeone, J. A., and Cowger, C. 1999. Population genetics and host resistance. Pages 115-130 in: Septoria on Cereals: A Study of Pathosystems. H. M. Anderson, P. Bowyer, and J. A. Lucas, eds. CAB International, Wallingford, UK.
- Mundt, C. C., Sackett, K. E., Wallace, L. D., Cowger, C., and Dudley, J. P. 2009. Long-distance dispersal and accelerating waves of disease: Empirical relationships. Am. Nat. 173:456-466.
- Pscheidt, J. W., and Ocamb, C. 2000. Pacific Northwest Plant Disease Management Handbook. A Pacific Northwest Extension Publication: Oregon State University, Washington State University, and the University of Idaho.
- Pscheidt, J. W., and Ocamb, C. 2005. Pacific Northwest Plant Disease Management Handbook. A Pacific Northwest Extension Publication: Oregon State University, Washington State University, and the University of Idaho, Corvallis, Oregon.
- QoI Working Group. 2016. QoI fungicides. Online publication. Fungicide Resistance Action Committee (FRAC). <http://www.frac.info/working-group/qol-fungicides>
- Siah, A., Elbekali, A. Y., Ramdani, A., Reignault, P., Torriani, S. F. F., Baggett, J. R., and Halama, P. 2014. QoI resistance and mitochondrial genetic structure of *Zymoseptoria tritici* in Morocco. Plant Dis. 98:1138-1144.
- Skinner, C. J., Holt, D., and Smith, T. M. F. 1989. Analysis of Complex Surveys. John Wiley & Sons, Chichester, UK.
- Skinner, W., Bailey, A., Renwick, A., Keon, J., Gurr, S., and Hargreaves, J. 1998. A single amino-acid substitution in the iron-sulphur protein subunit of succinate dehydrogenase determines resistance to carboxin in *Mycosphaerella graminicola*. Curr. Genet. 34:393-398.
- Thompson, W. T. 1975. Agricultural Chemicals Book IV—Fungicides. Thomson Publications, Fresno, CA.
- Torriani, S. F., Brunner, P. C., McDonald, B. A., and Sierotzki, H. 2009. QoI resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*. Pest Manage. Sci. 65:155-162.
- Van der Heyden, H., Dutilleul, P., Brodeur, L., and Carisse, O. 2014. Spatial distribution of single-nucleotide polymorphisms related to fungicide resistance and implications for sampling. Phytopathology 104:604-613.
- Zhan, J., Mundt, C. C., Hoffer, M. E., and McDonald, B. A. 2002. Local adaptation and effect of host genotype on the rate of pathogen evolution: An experimental test in a plant pathosystem. J. Evol. Biol. 15:634-647.