Reduced Virulence of Azoxystrobin-Resistant *Zymoseptoria tritici* Populations in Greenhouse Assays

Christina H. Hagerty and Christopher C. Mundt

Department of Botany and Plant Pathology, Oregon State University, 2082 Cordley Hall, 2701 SW Campus Way, Corvallis 97331.
Accepted for publication 9 April 2016.

ABSTRACT

Hagerty, C. H., and Mundt, C. C. 2016. Reduced virulence of azoxystrobin-resistant *Zymoseptoria tritici* populations in greenhouse assays. *Phytopathology* 106:884–889.

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 2 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.

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 U.S. \$9.91 billion 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 et al. 2000; Stammer et al. 2008). Furthermore, it takes an estimated U.S. \$250 million and 9 years of research and development to bring a new active ingredient to market (Phillips McDougall 2013). In the 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 et al. 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 with 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 et al. 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, because 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 used by farmers 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 arise 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 *M. 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 (C. H. Hagerty and C. C. Mundt, unpublished data). This is not surprising, given the biology and lifecycle of *Z. tritici* in the Willamette Valley (Mundt

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

<http://dx.doi.org/10.1094/PHYTO-01-16-0029-R>
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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 et al. 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” (Andrison 1995). Our findings indicate that azoxystrobin-resistant *Z. tritici* isolate mixtures are less 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. 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 7 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 (NWV), and the other trial was at the Hyslop Field Laboratory in South Willamette

Valley (SWV). 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 (NWV and SWV). 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 NWV and SWV 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 nonamended yeast malt agar (YMA) (Hardy Diagnostics) and one plate with YMA amended with technical-grade azoxystrobin (Syngenta) at 1 µg/ml. Three days after plating, isolate growth on the azoxystrobin-amended plate was compared with that of the nonamended plate. If the azoxystrobin-amended plate had reduced growth visually compared with the nonamended plate, the isolate was scored as sensitive. If the amended plate had the same growth visually compared with the nonamended 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 NWV and three fields from SWV. There was an average of approximately 150 km between the NWV and SWV 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 3 weeks after the final fungicide applications in the fields. The collections were phenotyped for resistance to azoxystrobin 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 (NWV and SWV), 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). In year 2, 10 isolates were used rather than 12, because the proportion of azoxystrobin 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 et al. 2008).

Greenhouse trials. Pathogen populations were evaluated during winter 2014 to 2015, and autumn 2015; for clarity and

TABLE 1. Analysis of variance for virulence of *Zymoseptoria tritici* isolate mixtures (12 isolates each) collected in the Willamette Valley of Oregon in 2012^a

Source of variation	df	MS	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	...	...

^a Each of two experiments consisted of a factorial of two inoculum mixture sources (North and South Willamette Valley) and two inoculum mixture phenotypes (azoxystrobin-resistant and -sensitive) evaluated in six replicates in the greenhouse. MS = mean squares.

TABLE 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^a

Source of variation	df	MS	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 collected–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	...	...

^a Each of two experiments consisted of a factorial of 2 years of collection (2012 and 2015) and two inoculum mixture phenotypes (azoxystrobin-resistant and -sensitive) evaluated in six replicates in the greenhouse. MS = mean squares.

TABLE 3. 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^a

Source of variation	df	MS	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 collected–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	...	...	...

^a Each of two experiments consisted of a factorial of 2 years of collection (2012 and 2015) and two inoculum mixture phenotypes (azoxystrobin-resistant and -sensitive) evaluated in six replicates in the greenhouse. MS = mean squares.

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 (NWV or SWV). In year 2, four inoculations occurred, two of which used SWV isolates and two of which used NWV isolates. The SWV treatments were a factorial of two pathogen phenotypes (azoxystrobin-sensitive or -resistant) times 2 years of collection from the SWV (2012 or 2015). The NWV treatments were identical to the SWV 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). 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/pot at seeding. In year 2, potting soil was amended with Miracle-Gro liquid fertilizer (Scott-Sierra Horticultural Products Co.) weekly

starting 2 weeks after seeding at a rate of approximately 100 ml/pot of the labeled dilution rate of 3.9 ml/liter. 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 ‘Skiles’ wheat (Flowers et al. 2010) were sown in each pot and seedlings were thinned to five plants per plot at approximately 10 days after planting. Skiles was developed primarily for eastern Oregon, and never attained dominance in the Willamette Valley. 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).

Inoculum preparation and inoculation. Isolates of *Z. tritici* were cultured individually for 4 days on fresh 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 10⁶ spores/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).

TABLE 4. 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^a

Source of variation	Experiment 1				Experiment 2			
	df	MS	F value	P value	df	MS	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	...	...

^a Each experiment was a factorial of 2 years of collection (2012 and 2015) and two inoculum mixture phenotypes (azoxystrobin-resistant and -sensitive) evaluated in six replicates. MS = mean squares.

Fig. 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 and “+” = resistant) and two source populations (“N” = North and “S” = South). Boxes encompass the second and third quartiles. Within boxes, horizontal lines are medians and dots are means. 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.

Fig. 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 and “+” = resistant) and two collection years (“12” = 2012 and “15” = 2015) in a greenhouse trial. Boxes encompass the second and third quartiles. Within boxes, horizontal lines are medians and dots are means. 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.

The treatment (75 ml) was 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.) for 96 h. Noninoculated 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 (version 0.98.1091; RStudio, Inc.). The *lm* function in the *lme4* package was used to build a linear model and analysis of variance (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 SWV isolate trial, experiments were combined and experiment was included as a cofactor because there were no significant ($P < 0.05$) interactions of experiment with main effects. For the year 2 NWV 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 (NWV versus SWV) did not have an effect on virulence ($P = 0.5530$) (Table 1). There were no significant interactions among sources of variation.

In the trial including only SWV 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). There were no significant interactions among sources of variation.

In the trial including only NWV experiments (year 2), experiments were analyzed separately, owing to significant experiment–year and experiment–year–isolate resistance interactions (Table 3). However, results from the two experiments were qualitatively similar, and

fungicide-sensitive inoculum was more virulent than fungicide-resistant inoculum in both experiments ($P < 0.0001$ for experiments 1 and 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 and 4).

Fig. 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 and “+” = resistant) and two collection years (“12” = 2012 and “15” = 2015) in a greenhouse trial. Boxes encompass the second and third quartiles. Within boxes, horizontal lines are medians and dots are means. 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.

Fig. 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 and “+” = resistant) and two collection years (“12” = 2012 and “15” = 2015), in **A**, greenhouse experiment 1 and **B**, experiment 2. Boxes encompass the second and third quartiles. Within boxes, horizontal lines are medians and dots are means. 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.

In all analyses, resistant inoculum was less virulent than sensitive inoculum (Figs. 1 to 4).

DISCUSSION

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 having no effect 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 (NWV versus SWV) or year of collection (2012 versus 2015). We used mixtures of 10 to 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 et al. 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 et al. 2011). These differences can be visualized in box plots between the 2 years (Figs. 1 to 3). In addition, mean square errors in the ANOVA were approximately half or less in the second year compared with the first year (Tables 1, 2, and 4). Nonetheless, the effect of azoxystrobin resistance on virulence did not change qualitatively between the 2 years of greenhouse studies.

Previous observations in both experimental field plots (Hayes et al. 2016) 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 et al. 1996; Stewart et al. 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. Stewart and McDonald (2014) obtained consistent pycnidial production in a greenhouse maintained at 70% humidity, whereas our greenhouse had no such humidity control. 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. Mixtures of resistant and sensitive isolates are currently being evaluated from field trials. 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 et al. 2008) to ameliorate virulence reductions of resistant phenotypes that were apparent in 2012. The hypothesis of compensatory mutations was rejected, because similar patterns in virulence between azoxystrobin-resistant and azoxystrobin -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 SWV 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 NWV trials. This result could be due to either differing selective pressures or to sampling effects between the 2 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 backgrounds 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 with 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 et al. 2009). The same is almost certain 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.

ACKNOWLEDGMENTS

This research was supported by the Oregon Agricultural Experiment Station. We thank K. E. Sackett, V. Le, and E. M. Sykes for technical help with inoculations, greenhouse assistance, and data collection; the Oregon State University greenhouse staff for controlling aphids and managing greenhouse facilities; and R. C. Graebner for general support.

LITERATURE CITED

- Andersson, D. I., and Levin, B. R. 1999. The biological cost of antibiotic resistance. *Curr. Opin. Microbiol.* 2:489-493.
- Andriveau, D. 1995. Nomenclature for pathogenicity and virulence: Precision vs. tradition. *Phytopathology* 85:518-519.
- Avila-Adame, C., and Köller, W. 2003. Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin. *Curr. Genet.* 42:332-338.
- Bergelson, J., and Purrington, C. B. 1996. Surveying patterns in the cost of resistance in plants. *Am. Nat.* 138:536-558.
- Brunner, P. C., Stefanato, F. L., and McDonald, B. A. 2008. Evolution of the CYP51 gene in *Mycosphaerella graminicola*: Evidence for intragenic recombination and selective replacement. *Mol. Plant Pathol.* 9:305-316.
- Chin, K. M., Chavaillaz, D., Kaesbohrer, M., Staub, T., and Felsenstein, F. G. 2001. Characterizing resistance risk of *Erysiphe graminis* f. sp. *tritici* to strobilurins. *Crop Prot.* 20:87-96.
- Cools, H. J., and Fraaije, B. A. 2008. Are azole fungicides losing ground against Septoria wheat disease? Resistance mechanisms in *Mycosphaerella graminicola*. *Pest Manage. Sci.* 64:681-684.
- Cooper, J., and Dobson, H. 2007. The benefits of pesticides to mankind and the environment. *Crop Prot.* 26:1337-1348.
- Délye, C., Y. Menchari, S. Michel, É. Cadet, and V.L. Corre. 2013. A new insight into arable weed adaptive evolution: Mutations endowing herbicide resistance also affect germination dynamics and seedling emergence. *Ann. Bot.* 111:681-691.
- DiLeone, J. A., Karow, R. S., Mundt, C. C., and Coakley, S. M. 1997. Biology and control of the septoria diseases of winter wheat in western Oregon.

- Online publication. Agricultural Experiment Station, Oregon State University, Corvallis. <http://ir.library.oregonstate.edu/xmlui/handle/1957/41545>
- Drabešová, 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.
- 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.
- Eyal, Z. 1987. The Septoria Diseases of Wheat: Concepts and Methods of Disease Management. CIMMYT, Mexico.
- Flowers, M. D., Peterson, C. J., Hulting, A. G., Burns, J. W., Guy, S. O., and Kuehner, J. 2010. Skiles: Soft white winter wheat. Online publication. Extension Service, Oregon State University, Corvallis. <http://hdl.handle.net/1957/15300>
- Fones, H., and Gurr, S. 2015. The impact of *Septoria tritici* blotch disease on wheat: An EU perspective. *Fungal Genet. Biol.* 79:3-7.
- 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. 2005. FRAC code list. Online publication. <http://www.google.com/patents/US7528232>
- Gagneux, S., Long, C. D., Small, P. M., Van, T., Schoolnik, G. K., and Bohannan, B. J. M. 2006. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. *Science* 312:1944-1946.
- Gisi, U., Sierotzki, H., Cook, A., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manage. Sci.* 58:859-867.
- Grasso, V., Palermo, S., Sierotzki, H., Garibaldi, A., and Gisi, U. 2006. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Manage. Sci.* 62:465-472.
- Gubbins, S., and Gilligan, C. 1999. Invasion thresholds for fungicide resistance: Deterministic and stochastic analyses. *Proc. R. Soc. B Biol. Sci.* 266:2539-2549.
- Hagerty, C. H., and Mundt, C. C. 2015. *Zymoseptoria tritici* fungicide resistance dynamics: Seasonal changes and effects on virulence. Page 41 in: Resistance 2015. Rothamsted Research, Harpenden, Hertfordshire, UK.
- Hayes, L.E., K.A. Sackett, N.P. Anderson, M.D. Flowers, and C.C. Mundt. 2016. Evidence of selection for fungicide resistance in *Zymoseptoria tritici* populations on wheat in western Oregon. *Plant Dis.* 100:483-489.
- Hirooka, T., and Ishii, H. 2013. Chemical control of plant diseases. *J. Gen. Plant Pathol.* 79:390-401.
- Jeger, M. J., Wijngaarden, P. J., and Hoekstra, R. F. 2008. Adaptation to the cost of resistance in a haploid clonally reproducing organism: The role of mutation, migration and selection. *J. Theor. Biol.* 252:621-632.
- Knight, S. C., Anthony, V. M., Brady, A. M., Greenland, A. J., Heaney, S. P., Murray, D. C., Powell, K. A., Schulz, M. A., Spinks, C. A., and Worthington, P. A. 1997. Rationale and perspectives on the development of fungicides. *Annu. Rev. Phytopathol.* 35:349-372.
- Leadbeater, A. 2015. Recent developments and challenges in chemical disease control. *Plant Prot. Sci.* 51:163-169.
- Levy, Y., Levi, R., and Cohen, Y. 1983. Buildup of a pathogen subpopulation resistant to a systemic fungicide under various control strategies: A flexible simulation model. *Phytopathology* 73:1475-1480.
- Linde, C. C., Zhan, J., and McDonald, B. A. 2002. Population structure of *Mycosphaerella graminicola*: From lesions to continents. *Phytopathology* 92:946-955.
- Lipsitch, M., Siller, S., and Nowak, M. A. 1996. The evolution of virulence in pathogens with vertical and horizontal transmission. *Evolution* 50:1729-1741.
- Ma, B., and Uddin, W. 2009. Fitness and competitive ability of an azoxystrobin-resistant G143A mutant of *Magnaporthe oryzae* from perennial ryegrass. *Plant Dis.* 93:1044-1049.
- Menchari, Y., Chauvel, B., Darmency, H., and Délye, C. 2008. Fitness costs associated with three mutant acetyl-coenzyme A carboxylase alleles endowing herbicide resistance in black-grass *Alopecurus myosuroides*. *J. Appl. Ecol.* 45:939-947.
- Mikaberidze, A., and McDonald, B. A. 2015. Fitness cost of resistance: Impact on management. Pages 77-89 in: Fungicide Resistance in Plant Pathogens. H. Ishii and D. W. Hollomon, eds. Springer, Tokyo, Japan.
- Milgroom, M. G., and Fry, W. E. 1988. A simulation analysis of the epidemiological principles for fungicide resistance management in pathogen populations. *Phytopathology* 78:565-570.
- 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*. CAB International, Wallingford, UK.
- Phillips McDougall. 2011. AgriService Report. Phillips McDougall, Midlothian.
- Phillips McDougall. 2013. R & D trends for chemical crop protection products and the position of the European market. Online publication. https://issuu.com/cropprotection/docs/r_and_d_study_2013_v1.8_webversion_
- Ponomarenko, A., Goodwin, S. B., and Kema, G. H. J. 2011. *Septoria tritici* blotch (STB) of wheat. Online publication. *Plant Health Instruct.* doi: 10.1094/PHI-I-2011-0407-01
- Russell, P. E. 2005. A century of fungicide evolution. *J. Agric. Sci.* 143:11-25.
- Shaw, M. W. 1989. Independent action of fungicides and its consequences for strategies to retard the evolution of fungicide resistance. *Crop Prot.* 8:405-411.
- Shaw, M. W. 1993. Theoretical analysis of the effect of interacting activities on the rate of selection for combined resistance to fungicide mixtures. *Crop Prot.* 12:120-126.
- Shaw, M. W. 2006. Is there such a thing as a fungicide resistance strategy? A modeller's perspective. *Asp. Appl. Biol.* 78:95-103.
- 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.
- Sierotzki, H., Wullschlegel, J., and Gisi, U. 2000. Point mutation in cytochrome b gene conferring resistance to strobilurin fungicides in *Erysiphe graminis* f. sp. *tritici* field isolates. *Pestic. Biochem. Physiol.* 68:107-112.
- Stammler, G., Carstensen, M., Koch, A., Semar, M., Strobel, D., and Schlehuber, S. 2008. Frequency of different CYP51-haplotypes of *Mycosphaerella graminicola* and their impact on epoxiconazole-sensitivity and -field efficacy. *Crop Prot.* 27:1448-1456.
- Statistica. 2015. Global leading wheat producing countries 2015/2016 | Statistic. Online publication. Statista. <http://www.statista.com/statistics/237912/global-top-wheat-producing-countries/>
- Stewart, A. D., Logsdon, J. M., and Kelley, S. E. 2005. An empirical study of the evolution of virulence under both horizontal and vertical transmission. *Evolution* 59:730-739.
- Stewart, E. L., and McDonald, B. A. 2014. Measuring quantitative virulence in the wheat pathogen *Zymoseptoria tritici* using high-throughput automated image analysis. *Phytopathology* 104:985-992.
- 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 den Bosch, F., Oliver, R., van den Berg, F., and Paveley, N. 2014. Governing principles can guide fungicide-resistance management tactics. *Annu. Rev. Phytopathol.* 52:175-195.
- Vila-Aiub, M. M., Neve, P., and Powles, S. B. 2009. Fitness costs associated with evolved herbicide resistance alleles in plants. *New Phytol.* 184:751-767.
- Zhan, J., and McDonald, B. A. 2013. Experimental Measures of Pathogen Competition and Relative Fitness. *Annu. Rev. Phytopathol.* 51:131-153.