Baseline and Temporal Changes in Sensitivity of *Zymoseptoria tritici* Isolates to Benzovindiflupyr in Oregon, U.S.A., and Cross-Sensitivity to Other SDHI Fungicides

Christina H. Hagerty,^{1,†} Ann M. Klein,¹ Catherine L. Reardon,² Duncan R. Kroese,¹ Caroline J. Melle,² Kaci R. Graber,¹ and Christopher C. Mundt³

¹ Columbia Basin Agricultural Research Center, Oregon State University, Adams, OR 97810

² Soil and Water Conservation Unit, United States Department of Agriculture–Agricultural Research Service, Adams, OR 97810

³ Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97330

Abstract

Zymoseptoria tritici is the causal agent of Septoria tritici blotch (STB), a disease of wheat (*Triticum aestivum*) that results in significant yield loss worldwide. *Z. tritici*'s life cycle, reproductive system, effective population size, and gene flow put it at high likelihood of developing fungicide resistance. Succinate dehydrogenase inhibitor (SDHI) fungicides (FRAC code 7) were not widely used to control STB in the Willamette Valley until 2016. Field isolates of *Z. tritici* collected in the Willamette Valley at dates spanning the introduction of SDHI (2015 to 2017) were screened for sensitivity to four SDHI active ingredients: benzovindiflupyr, penthiopyrad, fluxapyroxad, and fluindapyr. Fungicide sensitivity changes were determined by the fungicide concentration at which fungal growth is decreased by 50% (EC₅₀) values. The benzovindiflupyr EC₅₀ values increased significantly, indicating a reduction in sensitivity, following the adoption of SDHI fungicides in Oregon ($P < 0.0001$). Additionally, significant reduction in cross-sensitivity among SDHI active ingredients

was also observed with a moderate and significant relationship between penthiopyrad and benzovindiflupyr ($P = 0.0002$) and a weak relationship between penthiopyrad and fluxapyroxad ($P = 0.0482$). No change in cross-sensitivity was observed with fluindapyr, which has not yet been labeled in the region. The results document a decrease in SDHI sensitivity in *Z. tritici* isolates following the introduction of the active ingredients to the Willamette Valley. The reduction in cross-sensitivity observed between SDHI active ingredients highlights the notion that careful consideration is required to manage fungicide resistance and suggests that within-group rotation is insufficient for resistance management.

Keywords: cereals and grains, cultural and biological practices, disease management, field crops, fungi, fungicide resistance, Pacific Northwest, SDHI, Septoria tritici blotch, succinate dehydrogenase inhibitor, wheat, *Zymoseptoria tritici*

Septoria tritici blotch (STB), caused by the fungus *Zymoseptoria tritici* (synonym: *Mycosphaerella graminicola*), is a yield-limiting and economically significant disease of wheat. It is estimated that more than 50 million ha of wheat are affected by STB worldwide each year, largely in high-rainfall areas (Gilchrist and Dubin 2002). STB is the number one yield-limiting pathogen in Russia and western Europe and is the second most yield-limiting pathogen of wheat in the United States behind rust (McDonald and Mundt 2016; Ponomarenko et al. 2011). In particular, the Mediterranean climate of the Willamette Valley of Oregon, which experiences the majority of rainfall between autumn and spring (Taylor and Bartlett 1993), allows for STB to be an annually occurring and ubiquitous disease of local wheat crops.

STB control can be achieved through genetic resistance, cultural controls, and fungicides, although each approach has significant limitations (Torriani et al. 2015). Cultivars with genetic resistance are available; however, this resistance is often overcome due to rapid genetic changes in populations of *Z. tritici* (Brown et al. 2015; McDonald and Mundt 2016). Cultural controls, including rotation with nonhost plants and deep tillage of crop residue, can help break the disease cycle. However, these approaches can be ineffective due to the potential for long-distance dispersal of *Z. tritici* ascospores (Shaw and Royle 1989) and the increasingly common high-residue, no-tillage production systems that are being adopted by many farmers in the Pacific Northwest (PNW) region of the United States (Yorgey and Kruger

2017). The use of fungicides to control STB is generally considered the last resort due to the cost of application, potential for evolution of fungicide resistance, and environmental concerns. Nonetheless, the annual STB disease pressure in the Willamette Valley, and limited effectiveness and adoption of the cultural control methods discussed above, make seasonal fungicide applications necessary for STB control. This situation presents difficulties for fungicide resistance management in the region. STB fungicide resistance is a global challenge, with fungicide resistance having evolved independently in multiple wheat-producing regions, including the PNW (Chen and McDonald 1996; Torriani et al. 2009).

There are currently two main fungicide modes of action used for STB control: the demethylation inhibitors (DMIs), Fungicide Resistance Action Committee (FRAC) code 3, that have been used for decades, and the succinate dehydrogenase inhibitors (SDHIs), FRAC code 7. Previously, quinone outside inhibitors (QoIs), FRAC code 11, had been widely used to control STB, which resulted in the rapid evolution of resistance in European (Drabešová et al. 2013; Fraaije et al. 2003; Torriani et al. 2009) and U.S. *Z. tritici* populations (Estep et al. 2013). QoI resistance is conferred by a single point mutation in the gene encoding the mitochondrial cytochrome *b* (Sierotzki et al. 2000). The first report of QoI resistance occurred in the United Kingdom in 2001 (Fraaije et al. 2003). Resistance was initially observed in the Willamette Valley in 2012 (Estep et al. 2013) and, by 2016, 93% of *Z. tritici* isolates from the Willamette Valley were resistant to QoIs, thus rendering them useless for control of STB (Hagerty et al. 2017).

DMIs, including azole fungicides, were introduced in Europe in the late 1970s, with decreasing STB sensitivity observed in the last decade (Cools and Fraaije 2008; Stammer and Semar 2011). Since the occurrence and spread of resistance to QoIs in the early 2000s in Europe, DMIs have become the primary means for STB control. Sensitivity to DMIs is influenced by the haplotype of the CYP51 protein, the target of DMIs. New CYP51 haplotypes have emerged and

[†]Corresponding author: C. H. Hagerty; christina.hagerty@oregonstate.edu

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the frequency of less sensitive haplotypes in Europe has increased in recent years (Cools and Fraaije 2013; Huf et al. 2018; Stammer et al. 2008). Decreased sensitivity and eventual resistance to DMIs involves multiple mechanisms, including target-site mutation, gene overexpression, and enhanced efflux (Cools and Fraaije 2013). Complete DMI resistance has not been detected in the PNW but local research suggests that DMI efficacy has been reduced over time (Estep et al. 2015; Hagerty et al. 2017). Therefore, a key requirement for sustainable control and resistance management of STB is the continued availability of different STB control strategies.

SDHIs were labeled for use on wheat in Oregon in 2013 but they were not widely adopted for use until 2016 due to limited availability and high cost. SDHIs have primarily been used to control STB in the Willamette Valley, with some minimal use to control stripe rust. SDHIs tested in this study include technical-grade benzovindiflupyr (trade name: Solatenol; manufacturer: Syngenta), technical-grade penthiopyrad (trade name: Vertisan; manufacturer: DuPont), technical-grade fluxapyroxad (SDHI component of trade names: Nexicor and Priaxor; manufacturer: BASF), and technical-grade fluindapyr (trade name: unnamed/unreleased; manufacturer: FMC). Benzovindiflupyr, penthiopyrad, and fluxapyroxad are currently the only SDHIs used in North America. Release of fluindapyr by FMC is anticipated in 2020.

SDHIs target the succinate dehydrogenase (SDH) enzyme, which is composed of four subunits (A, B, C, and D) (Rehfuß et al. 2018) and is involved in the mitochondrial respiratory chain. SDHIs block the function of the enzyme by binding at the site normally occupied by ubiquinone, preventing its reduction to ubiquinol in the citric acid cycle. Nonsynonymous target-site mutations in the genes coding for the B, C, and D subunits of the SDH enzyme have been reported to confer resistance to SDHI in field-isolated *Z. tritici* (Rehfuß et al. 2018). Nontarget resistance has also been reported in *Z. tritici* isolates with a 519-bp insertion in the *MgMFS1* promoter region that upregulates expression of an efflux pump (Omrane et al. 2015). As a result, SDHIs are classified as a high to medium fungicide-resistance-risk compound (FRAC 2005).

SDHIs have been used to control STB in Europe since the release of boscalid in 2003, and SDHI resistance monitoring has been carried out since their introduction. Loss of *Z. tritici* sensitivity to SDHIs due to amino acid changes in the SDH enzyme was first reported in *Z. tritici* isolates from France and the United Kingdom in 2012, Germany in 2013, and Ireland in 2014 (FRAC SDHI Working Group 2013, 2015). The appearance of less sensitive *Z. tritici* isolates in Europe coincided with the release of second-generation SDHIs and an overall increase in SDHI use as a result of QoI resistance and superior *Z. tritici* control with SDHIs. New mutations associated with decreased

sensitivity in *Z. tritici* field isolates are reported almost yearly (Klappach et al. 2018).

Given the recent introduction of SDHIs to the PNW, the evolvability of *Z. tritici*, and the rapid appearance of resistance in Europe, it is critical to monitor STB in the PNW for SDHI resistance. Resistance monitoring is important for grower profitability, environmental sustainability, and to progress theory and understanding of practical fungicide resistance management. To address this need, we have collected *Z. tritici* isolates from the Willamette Valley, OR over 3 years spanning a timeframe pre- and post- release of SDHIs for use on cereal crops in Oregon. The collection includes 2015 early-season isolates (SDHI-naïve), 2015 late-season isolates (limited SDHI exposure), 2016 isolates (year of widespread release with open access to SDHI product), and 2017 isolates (post- SDHI product release). Solatenol was labeled for use in spring 2015; therefore, the early 2015 *Z. tritici* isolate collection was not exposed to this SDHI product. A limited amount of SDHI product became available to Willamette Valley growers in spring 2015, and would have been applied at flag leaf (Feekes 10) (Large 1954), resulting in limited SDHI exposure. In field season 2016, QoI-DMI blends were likely applied on a widespread basis for early-season stripe rust control, which inadvertently placed selective pressure on STB infections present in the field. Some applications in field season 2017 may have included an SDHI, depending on the value of the crop and price of the fungicide.

Using the *Z. tritici* collections described above, we addressed the following research objectives for the Willamette Valley of Oregon: (i) establish the baseline sensitivity of *Z. tritici* to benzovindiflupyr, (ii) determine whether *Z. tritici* sensitivity to benzovindiflupyr changed following its introduction to the Willamette Valley of Oregon, and (iii) evaluate cross-sensitivity of *Z. tritici* between different SDHI compounds.

Materials and Methods

Field collections. Wheat leaves with visible STB pycnidia were collected in 2015 to 2017 from commercial wheat fields in the Willamette Valley of western Oregon using the same protocol detailed by Hagerty et al. (2017). Briefly, leaves were collected from commercial wheat fields in January 2015, May 2015, January 2016, and January 2017. Nine fields were sampled in the 2015 early collection, 17 fields were sampled in the 2015 late collection, 16 fields were sampled in the 2016 collection, and 10 fields were sampled in the 2017 collection. Collections were made from fields in 2015 based on presence of STB, physical accessibility, and grower cooperation. In subsequent field collections, the “next nearest” available wheat fields were sampled by walking two 300-m transects separated by a 300-m distance. Five leaves with at least one *Z. tritici* lesion containing pycnidia were collected at 30-m intervals along each of the two transects, for a total of 20 sample points within each field and 100 leaves collected from each field. For early-season collections occurring in January 2015, 2016, and 2017, the youngest leaf with pycnidia was chosen, which meant that third or fourth nodes were collected (Large 1954). For late-season collections occurring in May 2015 (approximately 3 weeks after final fungicide application), only flag leaves were collected. Leaves were pressed flat, dried, and stored at 4°C in a low-humidity cold room.

Fungal isolations and fungicide assays. One of the five leaves from each of the 20 transect intervals per field was removed from the cold room and placed on moistened filter paper at room temperature overnight. Extruded pycnidiospores were collected from individual pycnidia on symptomatic leaves and transferred to yeast mannitol agar (YMA) plates amended with gentamycin (10 ml liter⁻¹) and incubated in the dark at 22°C. Secondary transfers were dilution streaked to generate individual *Z. tritici* colonies. Tertiary transfers were made from individual *Z. tritici* colonies. Agar plugs were taken of individual colonies from the tertiary transfer plates and stored in 50% glycerol at -80°C. If the first of five leaves from each transect interval was not successful in establishing a pure culture, the process was repeated with the remaining four leaves until one pure culture was established per transect interval, for a total of 20 *Z. tritici* isolates per field.

Fig. 1. Incidence of tolerant isolates per field across collection dates. The number of Willamette Valley *Zymoseptoria tritici* isolates per field collection that were able to grow in benzovindiflupyr succinate dehydrogenase inhibitor fungicide at 10 mg liter⁻¹. Each point represents the number of tolerant isolates (out of 20 samples) for an individual field at a given sampling time. Points have been jittered for ease of visualization.

All isolates ($n = 797$) were screened for sensitivity to benzovindiflupyr. A subset of 64 isolates varying in sensitivity to benzovindiflupyr were selected and screened for sensitivity to three additional, related pyrazole-carboxamide SDHI chemicals: penthiopyrad, fluxapyroxad, and fluindapyr.

The 64-isolate subset for cross-sensitivity testing was chosen from the 2015 to 2017 collections based on results of the benzovindiflupyr assay: the 9 isolates with the highest benzovindiflupyr fungicide concentration at which fungal growth is decreased by 50% (EC_{50}) values and the 9 isolates with the lowest benzovindiflupyr EC_{50} values were chosen, and the remaining 46 isolates were chosen randomly from the remaining collection. Sensitivity of the *Z. tritici* isolates to these SDHI fungicides was assayed using a microplate method (Sykes 2015). Isolates were removed from glycerol storage, transferred to

fresh gentamycin-amended (10 ml liter^{-1}) YMA Petri plates, and incubated in the dark for 72 h at 22°C . Spore suspensions of each isolate were prepared by transferring a small loopful of spore mass to sterile water (5 ml) and vortexing for approximately 15 s. Spore concentrations were diluted to approximately 10^6 spores ml^{-1} of sterile water. Diluted spore suspension ($20 \mu\text{l}$) was added to potato dextrose broth ($60 \mu\text{l}$) amended with fungicide ($20 \mu\text{l}$) in a 96-well microtiter plate. All four fungicide technical-grade materials were 99.9% pure fine granular. A stock solution consisted of 0.1 g of technical-grade fungicide in 9.9 ml of dimethyl sulfoxide. Serial dilutions were then created from the stock solution at eight concentrations: 500, 50, 5, 0.5, 0.05, 0.005, 0.0005, or 0 mg liter^{-1} . Each isolate was tested at each fungicide concentration (500, 50, 5, 0.5, 0.05, 0.005, 0.0005, or 0 mg liter^{-1}), with each plate containing 11 isolates and one uninoculated

Fig. 2. Distribution of Willamette Valley *Zymoseptoria tritici* values of benzovindiflupyr concentration of fungicide that reduced *Z. tritici* growth by 50% (EC_{50}). Each histogram is a sample collection time. Each bar of the histogram represents the quantity of isolates at a given EC_{50} . Mean EC_{50} is indicated with the black point and 95% confidence interval about the mean is indicated with the black horizontal line.

control at the eight fungicide concentrations. The assay was replicated for all 64 isolates on three separate microtiter plates. Plates were sealed with sterile film and incubated on a microplate shaker (250 rpm) in the dark at 22°C for 5 days. Fungal growth was measured by optical density at 595 nm using a microplate spectrophotometer (SpectraMAX M2; Molecular Devices, San Jose, CA, U.S.A.) following a 5-s shake step at 120 rpm.

Statistical analysis. All statistical analyses were conducted with R statistical computing software (R Core Team 2013). EC_{50} values relative to the nonfungicide-treated controls were measured via optical density. Optical densities were averaged across the triplicate plates and corrected for background absorbance by subtracting the averaged absorbance values of the uninoculated controls. EC_{50} values were calculated from the modeled upper asymptote of the dose

response curve for each isolate using the SSasympt function in R (R Core Team 2013). Data were log transformed for normality based on nonnormal distribution of EC_{50} data. Variability in the isolate sensitivity to benzovindiflupyr across the collection dates was determined using a one-way analysis of variance (ANOVA) with a posthoc Tukey's honestly significant difference test to determine pairwise differences. The benzovindiflupyr tolerance level was designated as an isolate's ability to grow in benzovindiflupyr at a concentration of 10 mg liter⁻¹, with an arbitrary threshold of 0.1 optical density (595 nm) as an indication of significant fungal growth. This arbitrary tolerance threshold level of 0.1 optical density was chosen by observing the increased number of isolates beyond that threshold in the later collection dates and our effort to statistically evaluate the apparent shift in isolates with increased fungal growth in

Fig. 3. Scatterplot of pairwise comparisons of succinate dehydrogenase inhibitor fungicide active ingredients benzovindiflupyr, penthiopyrad, fluxapyroxad, and fluindapyr concentration at which fungal growth is decreased by 50% (EC_{50}). Probability (P) and correlation (r) values are displayed. Each point represents the EC_{50} value for a single isolate.

the presence of fungicide. Collection times with significantly different quantities of tolerant isolates per field were identified using an ANOVA, with collection time as the fixed effect and posthoc least significant difference (LSD). Pearson's correlations of log-transformed EC₅₀ values were used to assess potential cross-sensitivity in each pairwise combination of the four fungicides on the subset of 64 isolates which were tested for sensitivity to all four fungicides.

Results

The number of benzovindiflupyr-tolerant isolates per field significantly increased as a function of collection time ($P < 0.0001$). The posthoc LSD test revealed an LSD of 3.31 tolerant isolates per field, with an increasing incidence in 2016, 2017, and 2015 collections, respectively (Fig. 1).

Within each collection date, the mean EC₅₀ values were significantly different ($F_{(3, 769)} = 33.55, P < 0.0001$). Specifically, EC₅₀ values were significantly greater ($P < 0.0001$) in the late collection of 2015 than the early collection of 2015. The maximum EC₅₀ value occurred in the 2016 collection date and was significantly greater than either 2015 collection date ($P < 0.0001$) but was not different than values observed in 2017 ($P = 0.1259$) (Fig. 2).

Positive correlations were observed between EC₅₀ values for benzovindiflupyr and penthiopyrad ($r = 0.4642, P = 0.0002, n = 56$) and between penthiopyrad and fluxapyroxad ($r = 0.2629, P = 0.0482, n = 55$). Of the monitored genotypes, no significant correlation was found between benzovindiflupyr and fluxapyroxad ($r = 0.0300, P = 0.8278, n = 53$). Fluindapyr EC₅₀ values were not correlated with any of the other fungicides tested: benzovindiflupyr ($r = -0.0925, P = 0.5187, n = 49$), fluxapyroxad ($r = -0.0242, P = 0.8647, n = 50$), or penthiopyrad ($r = -0.0351, P = 0.8051, n = 50$) (Fig. 3).

Discussion

Resistance of plant pathogens to fungicides is a global problem that threatens agricultural sustainability and food security. Tracking the emergence and progression of fungicide resistance as new chemicals become available and are adopted by producers is critical for long-term agricultural productivity. In the current study, *Z. tritici* sensitivity to SDHI fungicides was assessed prior to and immediately following the release of benzovindiflupyr for agricultural use on cereals in the Willamette Valley, OR. Cross-sensitivity of a subset of isolates to three additional SDHIs (penthiopyrad, fluxapyroxad, and fluindapyr) was also assessed.

Trivapro (Syngenta), Priaxor (BASF), and Vertisan (DuPont) all contain an SDHI fungicide component and were labeled for use on cereals in Oregon in spring 2014 and 2015 field seasons. Although these products were labeled for use starting in 2014, they were essentially unused until spring 2016 due to limited product availability in Oregon combined with the high cost. In spring 2016, three simultaneous factors rapidly increased use of SDHIs in the Willamette Valley of Oregon: (i) SDHI products became widely accessible for grower use, (ii) product rebates helped increase affordability of SDHI products, and (iii) Oregon State University and Washington State University trial data indicated the success of SDHI products for control of STB and stripe rust. By collecting samples of *Z. tritici* from STB-infected plants shortly before the introduction of SDHIs (early 2015) and in subsequent years, we were able to assess the rapid evolution of *Z. tritici* to reduced sensitivity to this group of fungicides in the PNW.

The prevalence and magnitude of *Z. tritici* reduced sensitivity to benzovindiflupyr increased following release and adoption of the fungicide for use on cereal crops in Oregon. Tolerance to benzovindiflupyr was measured in isolates collected prior to and following the release of the fungicide. The number of tolerant *Z. tritici* isolates increased from an average of 0.3 tolerant isolates per field in early 2015 to an average of 1.4 tolerant isolates per field in late 2015 (Fig. 1). The most drastic increase in tolerant isolates occurred in the 2016 collection, where the average number of tolerant isolates per field increased to 11.9 from 1.4, and subsequently plateaued to an average of 9.6 tolerant isolates per field in 2017 (Fig. 1).

These data indicate that less-sensitive *Z. tritici* isolates infected plants shortly after benzovindiflupyr was used on a widespread scale in 2016. Relative to the first year following release (2016), sensitivity did not decrease in the second year (2017). This result suggests that resistance-reduced sensitivity was conferred by a mutation present as a standing genetic variation in the population and that 1 year was insufficient time for those mutations to be selected, or that tolerant isolates were less fit to survive between seasons. The increased frequency of tolerant isolates does not directly relate to field performance but we expect, over time, that tolerance may increase and potentially reduce field performance of SDHIs.

Reduced cross-sensitivity between the three introduced SDHI formulations was observed in a subset of the field isolates. A moderate level of decreased cross-sensitivity was detected with penthiopyrad and benzovindiflupyr, and a weak level of lowered cross-sensitivity between penthiopyrad and fluxapyroxad. There was no reduction in cross sensitivity observed with the released SDHIs to fluindapyr, which has not yet been labeled for use in Oregon. Though alterations in cross-sensitivity detected shortly after the introduction of SDHIs was not robust, *Z. tritici* may develop more cross-sensitivity after the continued use of SDHIs. Careful consideration of cross-sensitivity among SDHIs is critical for fungicide resistance management and our results suggest that within-group rotation is insufficient for resistance management. Rather, between-group product rotation is necessary to prolong SDHI efficacy and limit the evolution of cross-resistance.

Next-generation carboxamide fungicides have been used for STB control in Europe since 2005 (Fraaije et al. 2012). Though several mutations conditioning reduced sensitivity have been observed in recent years, these mutations have remained at low frequency thus far (FRAC 2018). This is a slower pattern of evolutionary change for resistance than that seen with strobilurin fungicides in either Europe or Oregon (Hagerty et al. 2017). Thus, control of STB by SDHI fungicides may not be in immediate danger of failure in Oregon. Nonetheless, our results are concerning for use of SDHIs for STB control in the Willamette Valley and warrant continued management to preserve this valuable resource. The first priority for fungicide resistance management is to reduce the necessity of fungicides by planting resistant cultivars, utilizing methods to reduce inoculum, rotating cereals with nonhost crops, and avoiding sites with high disease pressure (Staub 1991). Additionally, fungicide resistance of *Z. tritici* could also be driven by using SDHIs to control other foliar pathogens (e.g., stem, leaf, and stripe rust; eyespot; and sharp eyespot). Incidental exposure of *Z. tritici* to SDHIs could select for resistant populations even in areas where STB is not of concern. A similar phenomenon was observed in the mid-2000s when an aggressive race of stripe rust began infecting winter wheat in the Willamette Valley, prompting an increase in the use of strobilurin fungicides to control the rust. As a consequence, other potential pathogens, including *Z. tritici*, were exposed to strobilurins, leading to strobilurin resistance in *Z. tritici* (Estep et al. 2015; McDonald and Mundt 2016). If an SDHI fungicide application is determined to be necessary, resistance management methods, including judiciousness in application dose and frequency, appropriate timing of applications, and varying modes of action, should be employed (van den Bosch et al. 2014).

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