Baseline Sensitivity of *Ascochyta rabiei* to Azoxystrobin, Pyraclostrobin, and Boscalid

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ABSTRACT

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Ascochyta rabiei, causal agent of Ascochyta blight on chickpea (*Cicer arietinum*), can cause severe yield loss in the United States. Growers rely on applications of fungicides with site-specific modes of action such as the quinone outside inhibiting (QoI) fungicides azoxystrobin and pyraclostrobin, and the carboximide fungicide boscalid, to manage disease. In all, 51 isolates collected prior to QoI fungicide registration and 71 isolates collected prior to boscalid registration in the United States were tested in an in vitro assay to determine the effective fungicide concentration at which 50% of conidial germination was inhibited (EC_{50}) for each isolate–fungicide combination. The effect of salicylhydroxamic acid (SHAM) on conidia of *A. rabiei* in the presence and absence of azoxystrobin also was assessed to determine whether the fungus is capable of using alternative respiration. Five of nine *A. rabiei* isolates tested had significantly higher ($P \leq 0.05$) EC_{50} values when SHAM was not included in media amended with azoxystrobin, indicating that *A. rabiei* has the potential to use alternative respiration to overcome fungicide toxicity in vitro. EC_{50} values of azoxystrobin and pyraclostrobin ranged from 0.0182 to 0.0338 $\mu\text{g/ml}$ and from 0.0012 to 0.0033 $\mu\text{g/ml}$, with mean values of 0.0272 and 0.0023 $\mu\text{g/ml}$, respectively. EC_{50} values of boscalid ranged from 0.0177 to 0.4960 $\mu\text{g/ml}$, with a mean of 0.1903 $\mu\text{g/ml}$. Establishment of these baselines is the first step in developing a monitoring program to determine whether shifts in sensitivity to these fungicides are occurring in the *A. rabiei* pathogen population.

Additional keywords: *Didymella rabiei*, fungicide resistance

Ascochyta blight, caused by the fungal pathogen *Ascochyta rabiei* (Pass.) Labr. (teleomorph, *Didymella rabiei* (Kovatsch.) Arx.), is a limiting disease of chickpea (*Cicer arietinum* L.) production throughout the world (18,27). In the United States and Canada, chickpea production has decreased in the last decade due to the devastating effects of Ascochyta blight, which is considered to be the most important disease affecting chickpea production in these regions (5,7,30). *A. rabiei* can spread quickly throughout chickpea fields, causing significant yield losses (5,30). Development of chickpea cultivars with durable resistance has been complicated by the presence of different pathotypes of *A. rabiei* (5,7). Therefore, growers rely on fungicide applications to manage the disease (27).

Two of the most widely used fungicide active ingredients for control of Ascochyta blight in the United States are azoxystrobin (Amistar or Quadris; Syngenta Crop Protection, Greensboro, NC) and pyraclostrobin (Headline; BASF Corporation, Research Triangle Park, NC). Azoxystrobin became available for use on chickpea in the 2002 growing season in areas of the United States where section 18 emergency exemptions were approved for control of Ascochyta blight on chickpea. In 2003, both azoxystrobin and pyraclostrobin received United States Environmental Protection Agency section 3 registrations prior to the growing season. These fungicides are classified as quinone outside inhibitors (QoI) and block electron transport at the quinol-oxidizing site of the cytochrome bc_1 complex (complex III) in the mitochondrial respiration chain (2,10). The site-specific mode of action of this chemistry increases the potential for fungicide resistance to develop in the target fungal populations. Several fungal pathogens are reported to have reduced levels of sensitivity to QoI fungicides due to single amino acid substitutions in the cytochrome b site (6,10,15,16,23).

The fungicide boscalid (Endura; BASF Corporation) was registered in 2003 on

chickpea for control of Ascochyta blight in the United States, but was not available for use by growers until the 2004 growing season. Boscalid is a novel chemistry in the carboximide group that acts at succinate-ubiquinone reductase (complex II) in the mitochondrial respiration pathway (3). Due to price constraints, this fungicide currently has limited use in chickpea production in the United States; however, the site-specific mode of action increases the likelihood that shifts in fungicide sensitivity will occur in the pathogen population if boscalid use on chickpea becomes more widespread in the future. The first report of fungal resistance to boscalid was published recently by Avenot and Michailides (1), in which *Alternaria alternata* isolates from pistachio (*Pistacia vera*) in California were found to be resistant.

Because growers rely heavily on fungicide applications to manage Ascochyta blight, it is important to determine whether the fungal population is changing in response to selection pressure. Isolates of *D. bryoniae*, a pathogen of cucurbits in the same genus as the teleomorph of *Ascochyta rabiei* (*D. rabiei*), have been reported to be resistant to the QoI fungicide azoxystrobin (20,29). Because of these reports and the history of QoI resistance in other pathogens, a baseline sensitivity level should be established to facilitate a monitoring program to detect shifts in sensitivity. According to Brent and Holloman (4), there are three reasons to conduct baseline fungicide sensitivity studies: (i) to develop and test an accurate, rapid, reproducible method for determining the degree of sensitivity of large numbers of field samples of major target fungi, so that such a method is readily available for any future monitoring that may be required; (ii) to obtain initial data regarding the range of sensitivity that exists in major target pathogens and major areas of use, to serve as a baseline against which any future measurements of sensitivity can be compared in order to reveal any possible shifts in sensitivity; and (iii) to detect any differences in sensitivity between samples that might, through the buildup of the less-sensitive components, lead to future resistance problems. Jutsum et al. (12) and Russell (26) stressed the importance of determining the range of sensitivities present in target pathogen populations prior to commercialization of the product. Estab-

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lishing a baseline for the carboximide fungicide, boscalid, is a proactive approach to fungicide resistance management and will allow pathogen sensitivity to be monitored if the chemistry use becomes more widespread on chickpea in the United States.

Previous research involving *in vitro* testing of fungi in the presence of respiration-inhibiting fungicides has indicated that some fungi are able to use an alternative respiration pathway to bypass complex III and IV in the mitochondrial respiration chain, allowing fungal spores to germinate even in the presence of high doses of certain fungicides (22,31,35). This phenomenon is observed only *in vitro*, and it is hypothesized that plant-produced flavones prevent the induction of alternative oxidase in nature, thus inhibiting alternative respiration (22,31). However, alternative respiration is still important because it may strongly impact results of *in vitro* assays, leading to inaccurate assessments of fungicide sensitivity *in vitro*. The chemical salicylhydroxamic acid (SHAM) is used in QoI *in vitro* fungicide testing to prevent fungal pathogens from using an alternative respiration mechanism (22). The ability of *A. rabiei* to use alternative respiration has not been reported.

The objectives of this research were to (i) determine whether *A. rabiei* isolates are capable of using alternative respiration during *in vitro* fungicide sensitivity assays and (ii) establish the baseline sensitivities of *A. rabiei* isolates to azoxystrobin, pyraclostrobin, and boscalid using isolates collected prior to exposure to QoI and boscalid fungicides.

MATERIALS AND METHODS

Collection of isolates of *A. rabiei*. Fifty-one *A. rabiei* isolates were obtained

from the Ascochyta collection in the United States Department of Agriculture–Agricultural Research Service collection in Pullman, WA (Table 1). These *A. rabiei* isolates were collected prior to the registrations of QoI fungicides and boscalid in the United States, and represent a true baseline group with no possible exposure to any QoI chemistry or boscalid. An additional 20 isolates of *A. rabiei* were used to establish the baseline for boscalid (Table 1); these isolates were collected prior to the use of boscalid in U.S. chickpea fields. Each isolate was preserved for long-term storage by plating 2 µl of conidial suspension onto individual plates of potato dextrose agar (PDA) (Difco Laboratories, Detroit) with Whatman no. 1 filter paper cut into small strips, sterilized, and placed on the agar surface. Each isolate was grown in a growth chamber for 14 to 21 days under a diurnal cycle (12 h of light and 12 h of dark) at 20 ± 2°C, at which time the filter paper was covered with mycelia. Filter papers were removed from the agar surface using sterile forceps and dried for approximately 18 h in a laminar flow hood. Filter papers were placed in sterile 15-ml centrifuge tubes; tubes were sealed with Parafilm and stored at –20°C.

Preparation of *A. rabiei* isolates for conidia germination assays. All *A. rabiei* isolates in all experiments were prepared using the following methods adapted from Pasche et al. (24). Sterile 0.05% Tween 20 (Sigma-Aldrich, St. Louis) was added to 7-day-old cultures of *A. rabiei* and the conidia were dislodged from the agar using a sterile glass rod. The resulting conidial suspension was adjusted to 2 × 10⁵ conidia/ml using a hemacytometer. A conidial suspension (100 µl) of each isolate was pipetted onto each of two replicate petri

plates (60 by 15 mm). Plates were held at 19°C for 18 h in the dark. Following incubation, 100 conidia per plate were visually assessed microscopically (×100 magnification) and evaluated for germination. A conidium was considered to be germinated if the germ tube was at least as long as the length of the conidium.

Effect of SHAM on conidia germination. The effect of SHAM (Sigma-Aldrich) at a concentration of 100 µg/ml on *A. rabiei* conidial germination was examined in a preliminary experiment. Ten isolates (AR401, AR402, AR418, AR430, AR477, AR604, AR660, AR666, AR668, and AR721) were selected randomly to test on PDA amended with SHAM at 100 µg/ml and nonamended PDA. Random selection of the isolates was done using the RAND function in Microsoft Excel 2003 software (Microsoft Corp., Redmond, WA). For this experiment, a stock solution of SHAM was prepared by adding 100 mg of SHAM for each 1 ml of methanol. The final concentration of both acetone and methanol in media amended with fungicide and nonamended media was 0.1% by volume. All amendments were filter sterilized and added to the autoclaved media after it had cooled to 55°C. The experiment was arranged as a completely randomized design (CRD) with two replicate plates of each isolate. The experiment was repeated once in an additional run, and data were analyzed using the general linear model procedure (PROC GLM) in SAS (version 8.2; SAS Institute, Inc., Cary, NC). Data from each run were analyzed separately first to compute variances, and a two-tailed *F* test for equality of variances was conducted to determine whether trials could be combined. In the combined analysis, the lack of significant (*P* ≤ 0.05) run and run–isolate interactions were used additionally to determine whether runs could be combined. If run or run–isolate interactions were not significant, then run was dropped from the model and an analysis of variance was calculated.

Effect of SHAM and azoxystrobin on conidia germination. Nine isolates (AR401, AR402, AR418, AR477, AR604, AR660, AR666, AR668, and AR721) were randomly selected and tested to compare the effect of azoxystrobin on *in vitro* conidial germination with and without the addition of SHAM to the media. This experiment was conducted to determine whether alternative respiration is induced in *A. rabiei* by the presence of a respiration-inhibiting fungicide such as azoxystrobin. Isolates were prepared for plating using the methods described previously, with the addition of technical-grade azoxystrobin (97.6% a.i.; Syngenta Crop Protection) to the media. A stock solution of azoxystrobin was prepared at a concentration of 100 mg/ml in acetone. Serial dilutions of the stock solution were prepared in acetone and conidia germination was as-

Table 1. Collection information for baseline isolates of *Ascochyta rabiei* from chickpea

Year ^a	Location	Isolates ^b
1983	Pullman, WA	AR465, AR468, AR471, AR477
1984	Genesee, ID	AR714, AR721
1984	Pullman, WA	AR439, AR441, AR444, AR445, AR456
1987	Genesee, ID	AR401, AR402, AR403, AR404, AR405, AR406, AR407, AR408, AR453
1987	Kendrick, ID	AR430, AR437
1987	Lapwai, ID	AR414, AR415, AR416, AR417, AR418, AR419, AR420
1987	Nez Pierce County, ID	AR409, AR410, AR411, AR413
1994	Genesee, ID	AR423, AR424, AR425, AR427, AR428
1995	Albion, WA	AR590
1995	Genesee, ID	AR588, AR598, AR601, AR604
1995	LaGrande, OR	AR625
1995	Steptoe, WA	AR666, AR668
1995	Waitsburg, WA	AR616, AR617
1995	Walla Walla WA	AR618, AR660, AR661
2002	Fresno, CA	C2-1, C2-2, C2-4
2002	Genesee, ID	B3-15, B3-25, B3-45
2002	Pullman, WA	A2-25, A3-15, A4-15, CAB01-4
2003	Culdesac, ID	03-C1-3
2003	Genesee, ID	03-A1, 03-A2, 03-A3, 03-B1, 03-B2, 03-B3, 03-E3, 03-F1, 03-F4

^a Year collected. All isolates were used as boscalid fungicide baseline isolates, but only isolates collected prior to 2002 were used as azoxystrobin and pyraclostrobin fungicide baseline isolates.

^b Isolates with an “AR” designation were obtained from Dr. Frank Dugan, United States Department of Agriculture–Agricultural Research Service (USDA-ARS), Pullman, WA. All other isolates were obtained from Dr. Weidong Chen, USDA-ARS, Pullman, WA.

sessed on PDA amended with azoxystrobin at 0, 0.001, 0.01, 0.1, 1, and 10 µg/ml. Conidial germination also was assessed on PDA amended with the six concentrations of azoxystrobin and SHAM at 100 µg/ml dissolved in methanol. All amendments were filter sterilized and added to the autoclaved media after it had cooled to 55°C. Conidia were incubated and percent conidial germination was assessed as described previously. Conidial germination for each of the replicate plates was converted to percent inhibition compared with the untreated control by: $100 - ([\text{percent germination of fungicide-amended}] / [\text{mean percent germination of non-amended}])$. The fungicide concentration that effectively inhibited conidial germination by 50% of the untreated control (EC_{50}) was determined for each isolate by linear interpolation using the two concentrations that bracketed 50%. This experiment was arranged as a CRD with two replicate plates per isolate, and the experiment was repeated once in an additional run. Data were analyzed using PROC GLM in SAS (version 8.2) as described in the previous section. Least square means t tests (PDIF option in SAS) were used to compare EC_{50} values of individual *A. rabiei* conidia on SHAM-amended PDA versus nonamended PDA.

Determination of baseline EC_{50} values. Stock solutions of technical-grade formulations of azoxystrobin (97.6% a.i.; Syngenta Crop Protection), pyraclostrobin (99% a.i.; BASF Corporation), and boscalid (95% a.i.; BASF Corporation) were prepared at concentrations of 100 mg/ml in acetone. Serial dilutions in acetone were prepared for each fungicide. Fungicide sensitivity was determined by evaluating *A. rabiei* conidial germination on PDA amended with each fungicide at 0, 0.001, 0.01, 0.1, 1, and 10 µg/ml and SHAM at 100 µg/ml dissolved in methanol. The final concentration of both acetone and methanol in media amended with fungicide and nonamended media was 0.1% by volume. All amendments were filter sterilized and added to the autoclaved media after it had cooled to 55°C.

A. rabiei cultures were prepared for fungicide sensitivity testing using the methods described in the previous sections. Conidial germination and conversion of germination to percent inhibition was assessed and determined as described previously. EC_{50} values were determined for each isolate and fungicide.

Baseline isolates were tested across seven trials due to time and space constraints. In all, 6 to 12 isolates were tested in each trial along with an internal control isolate (AR666) that was used to determine reproducibility of the trials. An assay reproducibility test described by Wong and Wilcox (32) was used to validate the reproducibility of each of the seven trials conducted. For this reproducibility test, the

internal control isolate (AR666) was tested in another experiment that was repeated 10 times in different runs, and the assay reproducibility calculations used by Wong and Wilcox (32) were applied to the resulting EC_{50} values, in which the mean, standard error, and 95% confidence intervals were calculated for the internal control isolate. For each of the seven trials conducted to determine baseline EC_{50} values, if the EC_{50} value of the internal control isolate did not fall within the 95% confidence interval, then that specific trial was repeated until the internal control fell within the 95% confidence interval. Isolates were arranged in a CRD with two replicate plates per isolate. Each of the seven trials was repeated once over time in an additional run. A two-tailed F test was conducted as previously described to determine whether variances of the two runs were equal. In the combined analysis of the two runs, the lack of significant ($P \leq 0.05$) run and run-isolate interactions was used additionally to determine whether runs could be combined. If run or run-isolate interactions were not significant, then run was dropped from the model and an analysis of variance was calculated. The baseline sensitivity distributions of each fungicide were tested for normality using the Shapiro-Wilk test (PROC UNIVARIATE NORMAL) in SAS (version 8.2). Associations among baseline sensitivities of each fungicide were evaluated using Pearson correlation analysis (PROC CORR) in SAS (version 8.2).

RESULTS

Effect of SHAM on conidial germination. Analysis of the effects of SHAM on conidial germination indicated no significant isolate-SHAM interaction ($P = 0.3251$) and no effect of SHAM on *A. rabiei* conidial germination ($P = 0.4495$). Mean percent germination for conidia on PDA amended with 100 µg/ml of SHAM was 98.3%, compared with 98.5% for conidia on nonamended PDA. Because SHAM alone was determined not to influence conidial germination, it was used in subsequent trials.

Effect of SHAM and azoxystrobin on conidial germination. Separate analysis of experiments conducted to determine the impact of SHAM on conidial germination in the presence and absence of azoxystrobin produced equal variances according to an F test; therefore, experiments were combined for analysis. Analysis of EC_{50} values of the nine isolates exposed to azoxystrobin with and without SHAM at 100 µg/ml indicated that the main effects of isolate and SHAM were significant ($P = 0.0003$ and 0.0001 , respectively) and the interaction of isolate-SHAM was significant ($P = 0.0004$). In five of the nine isolates tested (AR401, AR402, AR418, AR668, and AR721), EC_{50} values were significantly ($P \leq 0.05$) greater when SHAM was not included in the fungicide-amended media (Table 2). No other significant differences were found.

Determination of baseline EC_{50} values. Analyses of in vitro fungicide sensitivity trials were conducted and the F test for homogeneity of variance indicated that variances were equal, and no significant ($P \leq 0.05$) interactions were observed between run and other factors. Therefore, data from runs were combined to determine the mean EC_{50} values for each isolate-fungicide combination. The range of EC_{50} values for isolates exposed to azoxystrobin was 0.0182 to 0.0338 µg/ml, and the mean value was 0.0272 µg/ml (Fig. 1). The azoxystrobin, EC_{50} values were normally distributed ($P = 0.0922$). For pyraclostrobin, the range of EC_{50} values of the isolates was 0.0012 to 0.0033 µg/ml, and the mean value was 0.0023 µg/ml (Fig. 2). The pyraclostrobin EC_{50} values were normally distributed ($P = 0.2787$). The range of EC_{50} values for isolates exposed to boscalid was 0.0177 to 0.4960 µg/ml, and the mean value was 0.1903 µg/ml (Fig. 3). The boscalid EC_{50} values did not have a normal distribution ($P = 0.0226$). Pearson correlation analysis indicated that there was a significant ($P = 0.0001$) relationship between azoxystrobin and pyraclostrobin baseline sensitivities ($r = 0.53$), and no other relationships were significant.

Table 2. Comparison of azoxystrobin effective concentration at which 50% of conidial germination was inhibited (EC_{50}) values (µg/ml) of nine *Ascochyta rabiei* baseline isolates in salicylhydroxamic acid (SHAM)-amended potato dextrose agar and nonamended potato dextrose agar

Isolate	SHAM-amended	Nonamended	<i>P</i> value ^a
AR401	0.0277	0.0509	0.0024
AR402	0.0287	0.0435	0.0237
AR418	0.0338	0.0566	0.0026
AR477	0.0251	0.0306	0.3307
AR604	0.0264	0.0314	0.3763
AR660	0.0335	0.0414	0.1728
AR666	0.0238	0.0340	0.0911
AR668	0.0209	0.0675	0.0001
AR721	0.0232	0.0399	0.0136
Mean	0.0269	0.0439	0.0003

^a P value for individual isolates were determined using least-square means t tests; P value for comparison of overall isolate means of SHAM-amended and nonamended was determined from an F test.

DISCUSSION

The chemical SHAM is used in QoI in vitro fungicide testing to prevent fungal pathogens from using an alternative respi-

ration mechanism to bypass complex III in the mitochondrial pathway (the QoI fungicide binding site), which may allow the fungus to germinate in the presence of

high levels of QoI fungicides (22,35). The effects of SHAM and azoxystrobin were tested with the pathogen *Alternaria alternata* (17). In that study, no significant differences were observed between EC_{50} values when SHAM was included with the fungicide and when it was omitted, although the mean EC_{50} value was 2× higher when SHAM was omitted than when SHAM was included in the fungicide-amended media (0.12 versus 0.06 µg/ml). In contrast, conidial germination of baseline isolates of *Pyricularia grisea* were inhibited by azoxystrobin and trifloxystrobin at fungicide concentrations of 0.1 µg/ml when SHAM was added to conidial suspensions at a rate of 100 µg/ml (31); however, when SHAM was not included in conidial suspensions, EC_{50} values of baseline isolates did not differ significantly from the EC_{50} values of resistant isolates (31). In vitro QoI fungicide resistance due to alternative respiration also has been demonstrated in *Venturia inaequalis* and *Septoria tritici* (22,35). The current study indicates that there are isolates in the *Ascochyta rabiei* population that may be able to use alternative respiration to bypass the QoI fungicide binding site, leading to higher in vitro EC_{50} values. These skewed EC_{50} values can, in turn, lead to inaccurate assessments of fungicide sensitivity in the pathogen population. Ziogas et al. (35), showed that alternative respiration occurs in both the wild-type and mutant strain of *S. tritici*. This information, along with our results, indicates that isolates need not be previously exposed to QoI fungicides to utilize alternative respiration. Therefore, SHAM at 100 µg/ml should be included in all in vitro QoI fungicide testing conducted with *A. rabiei*.

A. rabiei isolates exhibited a narrow range of EC_{50} values for azoxystrobin similar to other fungal pathogens with baselines previously established (Fig. 1). Baseline EC_{50} values of conidial isolates of *P. grisea* for azoxystrobin ranged from 0.015 to 0.064 µg/ml, with a mean of 0.0290 µg/ml (31). Similarly, in *Alternaria solani*, EC_{50} values for baseline isolates for azoxystrobin ranged from 0.011 to 0.090 µg/ml, with a mean of 0.038 µg/ml (24). Isolates of *Erysiphe graminis* f. sp. *tritici* also had similar values for azoxystrobin, ranging from 0.022 to 0.235 µg/ml (6).

Few examples exist in the literature reporting baseline sensitivity of fungal pathogens to pyraclostrobin. EC_{50} values for *A. solani* baseline isolates indicate that the fungus is 10 times more sensitive to pyraclostrobin than azoxystrobin (24), which is similar to the *Ascochyta rabiei* isolates tested in our research trials. Similarly, EC_{50} values for *Uromyces necator* sensitivity to pyraclostrobin ranged from 0.0016 to 0.010 µg/ml, with a mean of 0.0044 µg/ml (33), which is comparable with the EC_{50} values shown for *A. rabiei* in our study. In a study on fungicide sensitivity

Fig. 1. Frequency distribution of effective fungicide concentration at which 50% of conidial germination was inhibited (EC_{50}) values (µg/ml) for 51 baseline isolates of *Ascochyta rabiei* to azoxystrobin. Individual isolates are grouped in class intervals of 0.003 µg/ml; values on the X-axis indicate the midpoint of the interval.

Fig. 2. Frequency distribution of effective fungicide concentration at which 50% of conidial germination was inhibited (EC_{50}) values (µg/ml) for 51 baseline isolates of *Ascochyta rabiei* to pyraclostrobin. Individual isolates are grouped in class intervals of 0.0003 µg/ml; values on the X-axis indicate the midpoint of the interval.

Fig. 3. Frequency distribution of effective fungicide concentration at which 50% of conidial germination was inhibited (EC_{50}) values (µg/ml) for 71 baseline isolates of *Ascochyta rabiei* to boscalid. Individual isolates are grouped in class intervals of 0.05 µg/ml; values on the X-axis indicate the midpoint of the interval.

ties of citrus pathogens, Mondal et al. (17) reported baseline sensitivities of five fungal pathogens (*Colletotrichum acutatum*, *Alternaria alternata*, *Elsinoe fawcettii*, *Diaporthe citri*, and *Mycosphaerella citri*) to pyraclostrobin, and mean EC₅₀ values for isolate sensitivity to pyraclostrobin was over 8× higher for each of the five citrus pathogens than for *Ascochyta rabiei*. This could be attributed to the fact that Mondal et al. (17) used inhibition of mycelial growth to determine EC₅₀ values, rather than conidial germination inhibition. Because QoI fungicides are powerful inhibitors of spore germination (2), an assay based on spore germination is likely a better method for determining sensitivity of fungi to this chemistry. The difference in methodology could partially explain why baseline isolates of *A. rabiei* are more sensitive to pyraclostrobin than the citrus pathogens reported. A preliminary finding of resistance to azoxystrobin and pyraclostrobin in *Didymella rabiei* was reported in Canada (8). This report of QoI resistance in *D. rabiei* was based on mycelial growth inhibition without the addition of SHAM (8). A more definitive conclusion of the sensitivity of the Canadian isolates to QoI fungicides would be obtained if these isolates were additionally tested using conidial germination with the addition of SHAM and compared with the azoxystrobin baseline sensitivity developed. As discussed previously, our research indicates the importance of using SHAM when measuring *A. rabiei* sensitivity to QoI fungicides in vitro.

A few reports of in vitro fungal pathogen sensitivities to boscalid are available. In *Spilocaea oleagina*, EC₅₀ values for conidial germination of isolates exposed to boscalid ranged from 0.005 to 0.5 µg/ml, with a mean of 0.031 µg/ml (19). In *Alternaria solani*, EC₅₀ values of boscalid ranged from 0.275 to 2.70 µg/ml, with a mean of 0.6878 µg/ml (23). In *A. alternata*, EC₅₀ values of boscalid in isolates never before exposed to boscalid ranged from 0.089 to 3.435 µg/ml, with a mean of 1.515 µg/ml (1). Based on conidial germination of *Botrytis cinerea*, Stammler and Speakman (28) reported that EC₅₀ values of boscalid ranged from 0.01 to 0.21 µg/ml, with a mean of 0.06 µg/ml, while Zhang et al. (34) reported EC₅₀ values of 0.02 to 1.68 µg/ml, with a mean of 0.42 µg/ml. Zhang et al. (34) also reported EC₅₀ values of boscalid based on mycelial growth of *B. cinerea* ranging from 0.09 to 3.69 µg/ml, with a mean of 1.07 µg/ml. The comparison of conidial germination and mycelial growth of *B. cinerea* in the Zhang et al. study (34) indicated that conidial germination was more sensitive to boscalid than mycelial growth. Although these pathogens do not have similar mean EC₅₀ values compared with *Ascochyta rabiei* (0.1903 µg/ml), they do exhibit the same broad range in boscalid EC₅₀ values

displayed by individual *A. rabiei* isolates (0.0177 to 0.4960 µg/ml).

The EC₅₀ values for *A. rabiei* baseline isolates to azoxystrobin and pyraclostrobin were similar in that both had relatively narrow ranges in values, which were represented by two- and threefold differences in sensitivity to these fungicides for the majority of isolates, respectively (Figs. 1 and 2). Additionally, azoxystrobin and pyraclostrobin baseline sensitivities both were distributed normally, and there was a significant, positive relationship between azoxystrobin and pyraclostrobin sensitivity values. The narrow distribution of pyraclostrobin EC₅₀ values and 12-fold difference in mean EC₅₀ values when compared with azoxystrobin indicate that pyraclostrobin has higher intrinsic activity against *A. rabiei* than either azoxystrobin or boscalid (Figs. 1 to 3). This same phenomenon also has been found previously in both *Alternaria solani* and *U. necator*. In each case, isolates were 10 times more sensitive to pyraclostrobin than azoxystrobin as indicated by EC₅₀ values (24,33). The distribution of *Ascochyta rabiei* EC₅₀ values to boscalid had a broad range, which was represented by a 28-fold difference in sensitivity to this fungicide. This broad range of EC₅₀ values is a warning that the potential of *A. rabiei* developing resistance to boscalid is present (12).

Although information on fungicide sensitivity of ascospores of the teleomorph *D. rabiei* would be valuable for comparison purposes, *A. rabiei* conidia are the primary target of QoI fungicide applications to prevent repeated cycles of conidial infection. Conidia of *A. rabiei* often are considered to be secondary inoculum; however, conidia also can serve as primary inoculum by overwintering on infected debris and as inoculum on infected seed (9,13). The conidial stage of other ascomycetes has been used previously to assess QoI fungicide sensitivity in spore germination assays (6,21,29,31,33). Sensitivity tests of *Venturia inaequalis* isolates to flusilazole indicated that ascospores were more sensitive than conidia, but both were suitable for fungicide resistance monitoring (25). Fungicide sensitivities of *A. rabiei* conidia and ascospores were not compared in our research; however, using conidia of *A. rabiei* to establish baseline fungicide sensitivity levels and in future fungicide resistance monitoring should provide an accurate assessment.

Fungal plant pathogens that are able to generate variation through sexual recombination and that have a polycyclic disease cycle have an increased risk of developing resistance to fungicides (4,11,12,14). Sexual recombination occurs in *A. rabiei* (*D. rabiei*) and it has a polycyclic disease cycle. Due to these risk factors present in *A. rabiei*, and the high risk of resistance development in QoI and carboximide fungicides, baseline sensitivity developed in this

research will be important in monitoring *A. rabiei* populations to help ensure efficacy of current fungicide spray programs. A fungicide resistance *A. rabiei* monitoring program recently established at North Dakota State University, Fargo, is using these fungicide sensitivity baselines to measure for shifts in sensitivity of *A. rabiei* isolates exposed to these fungicides.

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