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Sensitivity of Kansas Isolates of *Sclerotinia homoeocarpa* to Boscalid

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Abstract

Seventy-one isolates of *Sclerotinia homoeocarpa* from 12 sites in Kansas were evaluated in vitro for sensitivity to boscalid using four-fold dilutions ranging from 0.00024 to 4.0 µg/ml to determine the effective concentration that inhibited growth by 50% (EC_{50}). The range of EC_{50} values was from 0.09 to 3.90 µg/ml with a mean of 0.77 µg/ml, and the $\log_{10}$ values were distributed normally. A subset of 26 isolates was also tested for boscalid sensitivity on fungicide-treated plants in a greenhouse assay. Higher in vitro EC_{50} values for boscalid were not correlated with higher percent relative disease severity on boscalid-treated plants. These results provide a starting point for further monitoring of boscalid sensitivity.

Introduction

Dollar spot, caused by *Sclerotinia homoeocarpa*, is a common disease of cultivated turfgrasses (5,21). The pathogen has a wide host range including both cool-season (C3) grasses such as creeping bentgrass (*Agrostis stolonifera*), Kentucky bluegrass (*Poa pratensis*), annual bluegrass (*Poa annua*), and fescues (*Festuca* spp.) as well as warm-season (C4) grasses such as bermudagrass (*Cynodon dactylon*), zoysiagrass (Zoysia spp.), and buffalograss (*Buchloë dactyloides*). Dollar spot is a frequent problem in creeping bentgrass putting greens and fairways in golf courses. In turfgrass cut at putting green height (2.5 to 4.0 mm), the disease causes tan, sunken infection centers that are 2 to 5 cm in diameter, and in higher-cut turf the infection centers usually range from 2 to 15 cm in diameter (5,21). Temperatures in the range from 15 to 30˚C are most favorable for disease development (21), but the disease can occur at temperatures outside that range, leading to a long period when control is required. In Kansas disease onset usually occurs in May or early June, decreases during the hot, mid-summer months, then reappears and becomes more severe from mid-August through September.

Dollar spot management in golf courses relies heavily on the application of fungicides, and more money is spent for control of dollar spot than any other turfgrass disease in the United States (23). Systemic fungicides in the methyl benzimidazole carbamate, demethylation inhibitor (DMI), succinate-dehydrogenase inhibitor (SDHI), and dicarboximide groups are labeled for dollar spot control, as is the contact material chlorothalonil (24).

Resistance of *S. homoeocarpa* to various fungicides has been reported in North America. Resistance to benzimidazoles was first reported in the United States in the 1970s in isolates from several eastern and midwestern states (25) and since then has been documented in numerous other locations (3,4,6,9,19). Resistance to DMI fungicides has also been documented (3,4,7,8,9,19). Boscalid is a newer fungicide that has been used to control dollar spot in many states, including Kansas (10,11,12,13). Boscalid is a pyridine-carboxamide fungicide in the SDHI class that inhibits respiration by disrupting complex II in the electron transport chain thus interfering with cellular energy production (2). The sensitivity to boscalid of several plant pathogenic fungi has been examined. Baseline sensitivities (sensitivities of isolates with no prior exposure to the

fungicide) have been established for *Botrytis cinerea* in greenhouse vegetables (17) and *Uncinula necator* in grapevine (26). High levels of fungicide resistance, including reduced fungicide performance on plant tissue, has been reported for *Alternaria alternata*, a pathogen of pistachio (1) and the cucurbit powdery mildew pathogen, *Podosphaera xanthii* (15). Field control failures have been reported in gummy stem blight of cucurbits caused by *Didymella bryoniae* (22). Field control failures or reduced efficacy on plants due to boscalid resistance have not yet been reported for dollar spot. An abstract of an in vitro baseline study (20) and one additional in vitro study of boscalid sensitivity of 58 *S. homoeocarpa* isolates from Massachusetts, Ohio, and Wisconsin have been published (18). There have been no studies on the boscalid sensitivity of *S. homoeocarpa* isolates from Kansas, and studies are lacking on comparisons of in vitro sensitivity and actual fungicide efficacy on plants. Since in vitro sensitivity differences may or may not correspond to differing fungicide performance on plants, it is important to compare in vitro results with studies on plants (16).

The objective of this study was to test the in vitro sensitivity of 71 Kansas isolates of *S. homoeocarpa* to boscalid and to compare the results to fungicide performance on plants for a subset of those isolates.

Collection, Isolation, and Storage of *Sclerotinia homoeocarpa*

Plugs of turfgrass, 3 to 10 cm in diameter, containing individual dollar spot infection centers were collected from 12 sites in nine counties in Kansas in 2007 and 2008 (Table 1). Infection centers collected from the same putting green were at least 1 m apart. *Sclerotinia homoeocarpa* was isolated from symptomatic leaf tissue by surface disinfecting in 0.6% sodium hypochlorite for 30 s, rinsing once in sterile water, blotting dry, and placing individual leaf blades on 9-cm-diameter petri plates containing ¼-strength potato dextrose agar (PDA), prepared by combining 6 g potato dextrose broth (Difco PDB, Benton, Dickinson and Company, Sparks, MD) and 15 g agar in 1 liter water. After 1 or 2 days incubation at 20 to 24°C, *S. homoeocarpa* growth was identified with the aid of a dissecting microscope based on morphological characteristics, and mycelium from one colony per infection center was transferred to water agar (WA, 15 g agar in 1 liter water). After 1 or 2 days incubation at 20 to 24°C, hyphal tips were transferred to ¼ PDA.

Millet grain inoculum was prepared as described previously (9,16). Briefly, 40 g millet seed was soaked overnight in 50 ml sterile water in a 250-ml Erlenmeyer flask, then autoclaved twice for 30 min (120°C, 138 kPa) within 24 h. After cooling, six 5-mm-diameter mycelial plugs from the margins of actively growing cultures were transferred to each flask. Millet cultures were incubated without shaking for 10 to 14 days at 20 to 24°C, dried in a laminar flow hood, then stored at -20°C until used.

Table 1. Source, year of collection, and prior exposure to boscalid of *Sclerotinia homoeocarpa* isolates used in this study.

Site	Turf stand information	Collection year(s)	Number of isolates	Boscalid exposure (no. of prior applications*)
AVPB	bentgrass green	2007	1	0
DCCC	bluegrass tees	2007	2	0
	bluegrass fairway	2007	1	0
RFTC	bentgrass green	2007	1	0
AVPV	bentgrass greens	2007	2	0
		2008	7	0
MCC	bentgrass greens	2007	1	N/A
		2008	6	
SRCC	bentgrass green	2008	1	1
CBHC	bentgrass greens	2008	10	N/A
NCLW	buffalograss lawn	2008	1	0
CHCC	bentgrass greens	2008	4	2
OSWT	bentgrass greens	2008	18	4
QRCC	bentgrass greens	2008	14	3
WCGC	bentgrass green	2007	1	4
	bluegrass fairway	2007	1	0
		Total	**71**	–

* Number of applications of boscalid prior to sampling at the site based on superintendents' spray records over 5 years prior to sampling. Boscalid was not available for turfgrass use prior to that time. N/A indicates that spray records were not available for that site.

In vitro Sensitivity

Seven isolates were tested four times in preliminary experiments to determine the reproducibility of the assay. Three to five days prior to the fungicide assay, one grain of colonized millet for each isolate was removed from the freezer and placed onto a 9-cm-diameter petri plate of WA. From this WA culture, 4-mm-diameter plugs from the colony margin were placed in the center of 9-cm-diameter plates of WA amended with boscalid (formulated as Emerald 70WG, 70% a.i., BASF Corp., Research Triangle Park, NC) at final active ingredient concentrations of 0.0, 0.00024, 0.00098, 0.0039, 0.016, 0.063, 0.25, 1.0, or 4.0 µg/ml (20). Cultures were incubated at 20 to 24°C for 5 days, at which time colony size was determined by measuring two perpendicular diameters and subtracting the size of the 4-mm-diameter plug. Percent relative growth was calculated as the ratio of the diameter on amended media to that on unamended media multiplied by 100 for each concentration. A dose-response curve for each of the seven preliminary isolates was plotted using the relative growth values and $\log_{10}$-transformed boscalid concentrations. The portion of the curve corresponding to the $\log_{10}$-tranformed concentrations of 0.0039, 0.016, 0.063, and 0.25 µg/ml was linear (Fig. 1). The percent relative growth was regressed against the $\log_{10}$-transformed concentrations, and the resulting equations were used to determine the $\log_{10}EC_{50}$ values (Excel 2003, Microsoft, Redmond, WA). The mean EC_{50} value for each isolate was calculated based on the four replications. The graphical representations of the data (Fig. 1) indicated that the assay was reproducible, and the remaining 64 isolates were then each tested twice, in two independent runs, at the same range of concentrations. The $\log_{10}EC_{50}$ value for each isolate was calculated as described above using the

linear portion of the dose-response curve, which sometimes varied slightly from the range that was linear for the seven preliminary isolates. Normality of the $\log_{10}EC_{50}$ values was tested using the Ryan-Joiner test (Minitab 15, Minitab Inc, State College, PA). For the isolates from sites where boscalid history was available, correlation between $\log_{10}EC_{50}$ values and number of prior exposures was calculated (Minitab 15, Minitab Inc.).

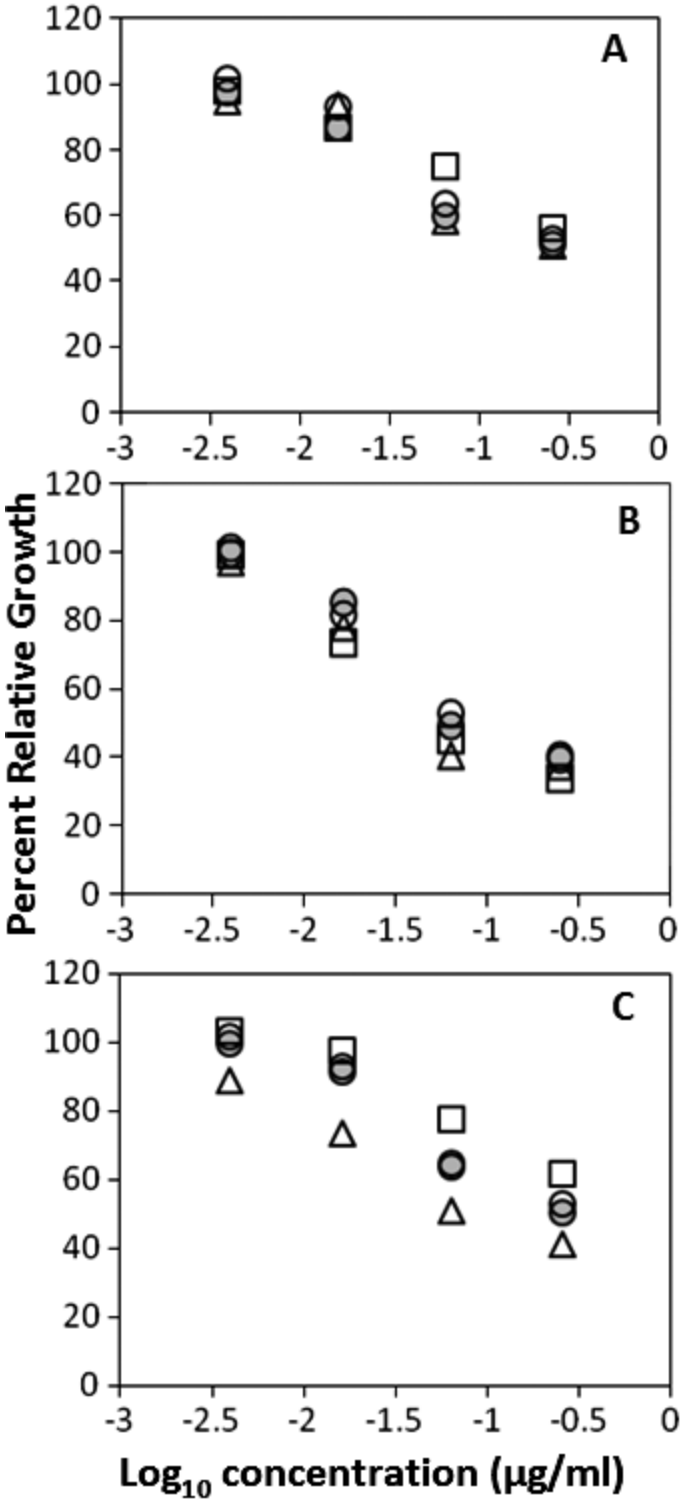

Fig. 1. Examples of dose response curves through the linear portion of the curve for three representative isolates (**A**, **B**, and **C**) of *Sclerotinia homoeocarpa* in preliminary boscalid sensitivity testing. Each isolate was tested four times (represented by the four symbols) at four-fold dilutions ranging from 0.00024 to 4.0 µg/ml of boscalid and an unamended control. Percent relative growth was calculated as the ratio of growth on amended media to that on unamended media multiplied by 100 for each concentration.

Across all 71 isolates, the range of EC_{50} values was from 0.09 to 3.90 µg/ml, representing approximately a 43-fold difference from the lowest to the highest, with a mean of 0.77 µg/ml. The $log_{10}EC_{50}$ values were normally distributed (Fig. 2) as determined by the Ryan-Joiner test. There was no significant linear correlation (P = 0.326) between in vitro sensitivity values and number of prior exposures to boscalid. In fact, the isolate with the highest EC_{50} value of 3.90 µg/ml was from a site that had not received any prior applications of boscalid.

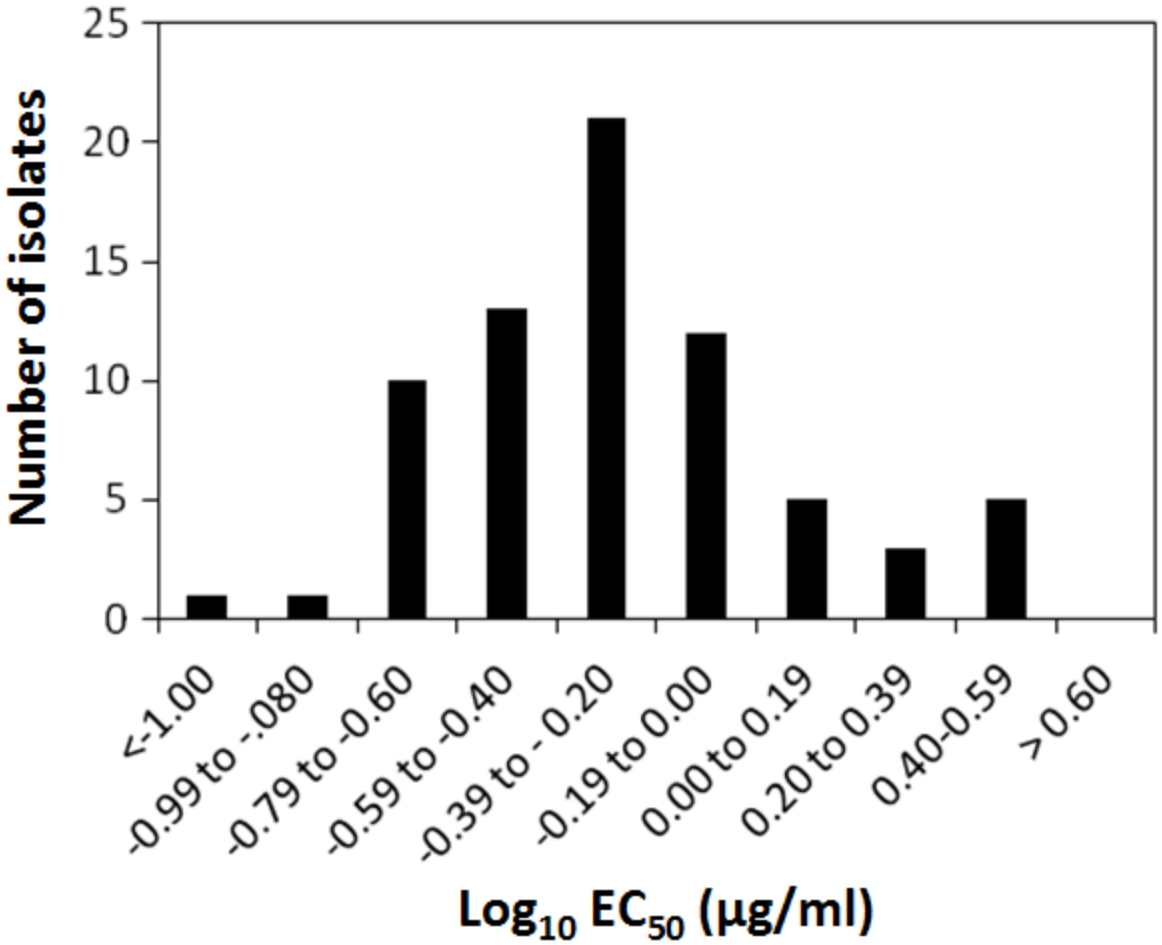

Fig. 2. Frequency distribution of the effective concentrations (log_{10}) of boscalid that inhibit mycelial growth by 50% (EC_{50}) for 71 isolates of *Sclerotinia homoeocarpa* collected from 12 sites in Kansas. Seven isolates were tested four times at concentrations ranging from 0.00024 to 4.0 µg/ml, and the remaining 64 isolates were each tested twice at those concentrations.

In a test of 82 baseline isolates, Scheidegger et al. (20) reported a mean EC_{50} of 0.09 µg/ml, a unimodal curve, and a range from 0.02 to 0.94 µg/ml. In the current study, in which some isolates had been previously exposed to boscalid, the mean EC_{50} was nearly 9 fold greater, 0.77 µg/ml, with a range from 0.09 to 3.90 µg/ml. It should be noted that technical grade boscalid was used in the baseline study (20), rather than formulated product (F. P. Wong, *personal communication*), which could potentially lead to differences. Wide ranges in EC_{50} values have been reported from studies of true baseline sensitivity in other fungi. Myresiotis et al. (17) studied the sensitivity of 55 baseline isolates of *Botrytis cinerea* and reported a mean EC_{50} value of 2.09 µg/ml and a range from 0.075 to 5.05 µg/ml, a 67-fold difference. Lu et al. (14) tested 41 baseline isolates of *Alternaria mali,* an apple pathogen, and the mean EC_{50} was 0.374 µg/ml with a 167-fold difference from the lowest to the highest value.

Determination of Discriminatory Concentration

Regression of the $log_{10}EC_{50}$ values with percent relative growth at 1.0, 0.25, and 0.063 µg/ml was conducted to determine a potential single discriminatory concentration for predicting EC_{50} values in a more streamlined assay (Minitab 15, Minitab Inc.). All regressions were significant ($P < 0.001$) and with R^2 values of 0.69, 0.76, and 0.62 for 1.0, 0.25, and 0.063 µg/ml, respectively (Fig. 3). Therefore, 0.25 µg/ml is recommended for future assays using a single concentration to predict $log_{10}EC_{50}$'s. This concentration was also found to be predictive of $log_{10}EC_{50}$ values in a study of baseline isolates [(20), F. P. Wong, *personal communication*].

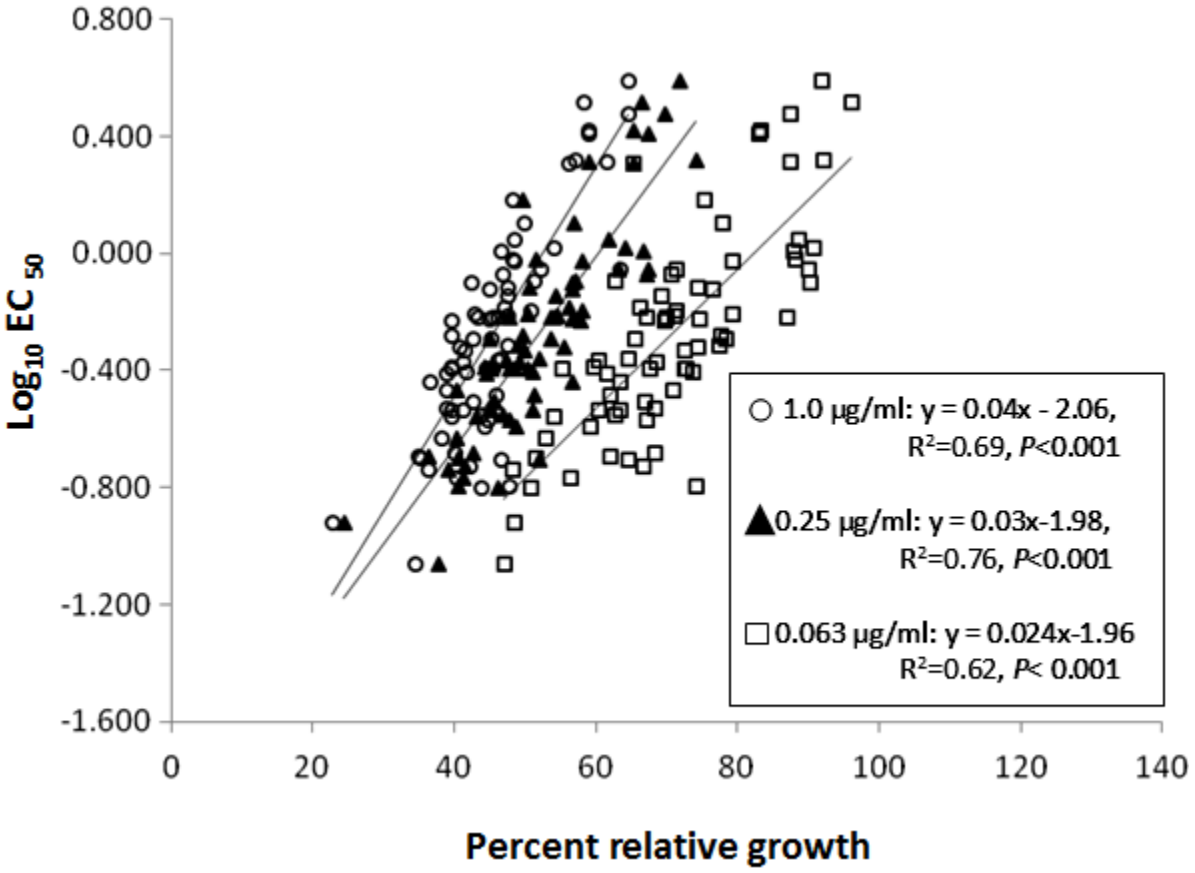

Fig. 3. Relationship between effective concentrations (log_{10}) of boscalid that inhibit mycelial growth by 50% (EC_{50}) for 71 isolates of *Sclerotinia homoeocarpa* and percent relative growth on water agar amended with boscalid at 1.0, 0.25, or 0.063 µg/ml. Seven isolates were tested four times at concentrations ranging from 0.00024 to 4.0 µg/ml, and the remaining 64 isolates were each tested twice at those concentrations.

Greenhouse Assays

Fungicide sensitivity was also determined in greenhouse assays using a subset of 26 isolates representing the full range of in vitro EC_{50} values from 0.09 to 3.89 µg/ml. For nine isolates, creeping bentgrass cultivar 'Crenshaw' was planted into 8 × 8 × 8-cm pots containing Metro-Mix 360, (Sun Gro Horticulture, Vancouver, Canada). Seventeen additional isolates were tested in 4-cm-diameter × 21-cm-deep pots. Pots were placed under intermittent mist for 10 days then placed in a greenhouse, and plant height was maintained at 2 cm by clipping with scissors three times per week. Assays were initiated five to six weeks after seeding.

Plants were treated with boscalid using a CO_2-powered hand-held spray boom equipped with one 8008EVS even-spray nozzle (Spraying Systems Co., Wheaton, IL) at 207 kPa in water equivalent to a field application of 825 liters/ha. Boscalid (formulated as Emerald, described above) was applied at 0.45, 0.11, and 0.03 mg a.i./ml, equivalent to the lowest labeled rate and 1/4

and 1/16 dilutions, respectively. Untreated checks were sprayed with water. After drying for 3 to 4 h, plants were inoculated by placing one colonized millet seed (described above) into each of the four quadrants of the pot for the larger pots and one seed at the center for the smaller pots. Sterile millet controls were included for each fungicide treatment. There were three replicate pots of each fungicide-isolate combination. Plants were misted lightly with water using a small CO_2-powered aerosol sprayer (Preval, Coal City, IL) and placed into plastic containers in the dark for 48 h at 24°C. The pots were then placed in a greenhouse for five daily cycles of 9 h ambient daylight conditions followed by misting, then 15 h overnight in the dark in moist chambers in the laboratory. After a final 48-h period in the greenhouse (9 days after inoculation), disease severity was assessed by visually estimating the percentage of blighted tissue per pot. For each isolate-fungicide combination, a percent relative disease severity score was calculated as the ratio of the disease severity in fungicide treatment to disease severity in the untreated control multiplied by 100. All isolates were tested twice. Correlation analysis (Minitab 15 Statistical Software, State College, PA) was conducted to examine potential relationships between the in vitro $\log_{10}EC_{50}$ values and the relative severity score in the greenhouse assays for each fungicide concentration.

Some *S. homoeocarpa* mycelium and dollar spot blighting developed in plants treated with all three rates of boscalid but at much lower severity than in the non-fungicide-treated controls, which always had at least 50% severity and often had > 80% severity. There was no significant correlation between in vitro $\log_{10}EC_{50}$ values and relative severity in the greenhouse assays for any of the fungicide rates ($P > 0.05$) (Fig. 4). While there was a 43-fold difference in the range of EC_{50} values in the in vitro assays, higher values did not correspond to any significant reduction in fungicide efficacy in plants. It is important to complement in vitro assays with experiments in plants. In a recent study of boscalid sensitivity, 58 *S. homoeocarpa* isolates from Massachusetts, Ohio, and Wisconsin had a reported mean EC_{50} value of 4.67 µg/ml and range from 1.02 to 21.38 µg/ml showing less in vitro sensitivity than the Kansas isolates, but no assays on plant tissue were performed (18).

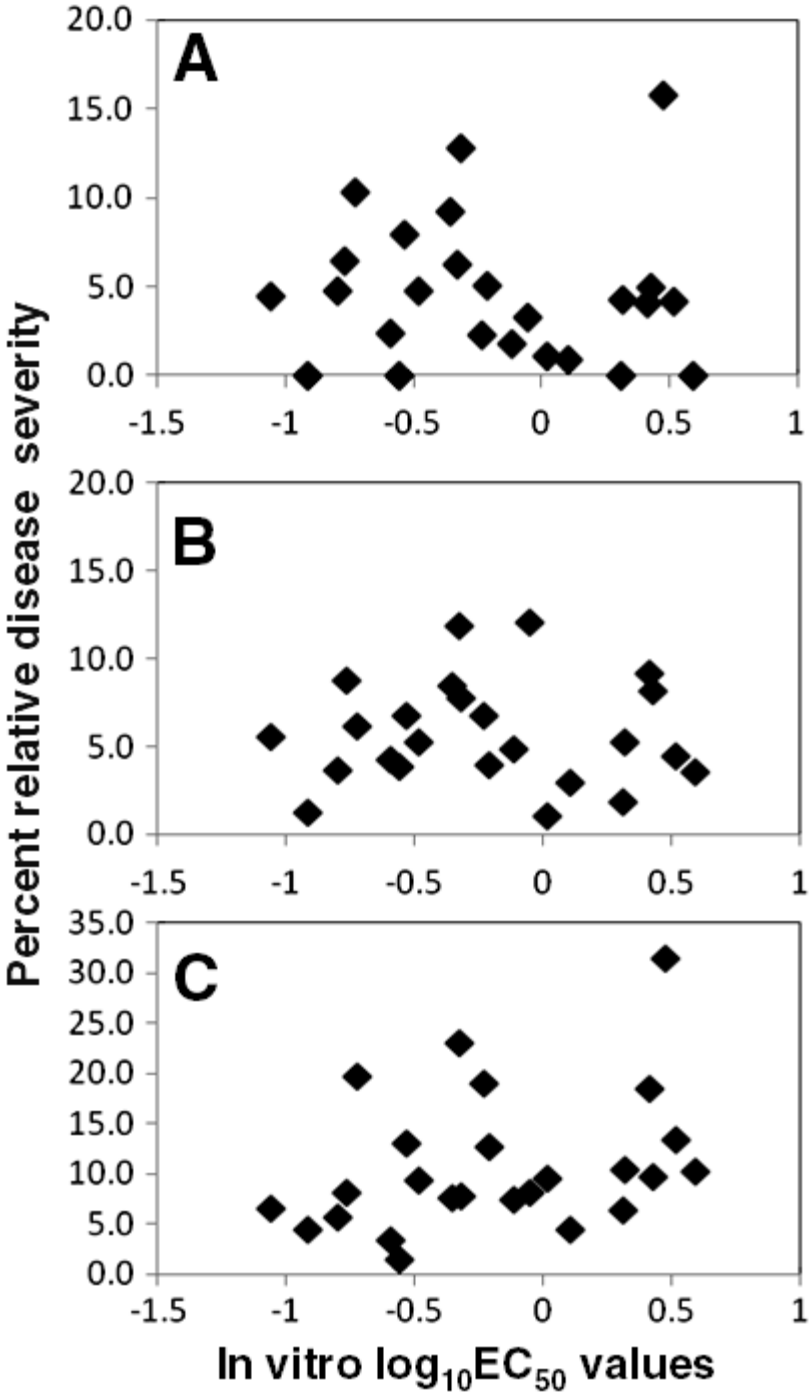

Fig. 4. Percent relative disease severity values in greenhouse assay for 26 isolates of *Sclerotinia homoeocarpa* inoculated onto plants treated with boscalid at 0.45 (**A**), 0.11 (**B**), or 0.03 (**C**) mg a.i./ml compared to in vitro $\log_{10}EC_{50}$ values of those isolates. To determine in vitro $\log_{10}EC_{50}$ values isolates were tested at concentrations ranging from 0.00024 to 4.0 µg/ml. Percent relative disease severity in greenhouse inoculations was calculated as the ratio of the disease severity in fungicide treatment to disease severity in the untreated control multiplied by 100. Correlations between percent relative disease severity and in vitro $\log_{10}EC_{50}$ values were not significant ($P > 0.05$) for any concentration of boscalid.

Fungal isolates with a high level of resistance (EC_{50} > 100 µg/ml) to boscalid have been detected in other pathosystems and field control failures or reduced efficacy on plants have been observed. Several *Alternaria alternata* isolates from a pistachio orchard with prior boscalid use had EC_{50} values > 100 µg/ml (1). In contrast, orchards with no prior use had a range from 0.89 to 3.435 µg/ml. Isolates of the cucurbit powdery mildew pathogen, *Podosphaera xanthii*, resistant to 175 µg/ml were detected in New York and Pennsylvania (15). None of the *S. homoeocarpa* isolates in the current study had extremely high EC_{50} values, such as > 100 µg/ml, nor were there any complete control failures in the greenhouse tests or any reductions in efficacy even at the lowest rate of boscalid.

Conclusions

This is the first study of boscalid sensitivity in isolates of *S. homeocarpa* from Kansas, and to our knowledge, the first comparison of in vitro sensitivity and in planta fungicide efficacy for boscalid for this pathogen. The assay using a full range of concentrations appears to be reliable and reproducible. In addition, a single discriminatory concentration of 0.025 µg/ml is predictive of $\log_{10}EC_{50}$ values for isolates in this range. If truly field-resistant isolates are encountered that do not fall in this pattern, the testing procedure can be further modified. Results from other pathosystems suggest that control failures are associated with extremely high EC_{50} values, such as >100 µg/ml. Though a wide range of in vitro EC_{50} values was observed, the differences did not correspond to reductions in fungicide efficacy in greenhouse tests, illustrating the importance of comparing in vitro results with testing on plants, especially when assays are being newly developed.

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