Differential Sensitivity to Boscalid in Conidia and Ascospores of *Didymella bryoniae* and Frequency of Boscalid-Insensitive Isolates in South Carolina

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Abstract

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Since 2003, a 2:1 mixture of the fungicides boscalid and pyraclostrobin (Pristine) has been used widely on watermelon and other cucurbits, primarily to control gummy stem blight caused by *Didymella bryoniae*. Several isolates of *D. bryoniae* that were insensitive to boscalid at 10 mg/liter were found in a watermelon research plot in South Carolina in 2008. In total, 201 isolates collected between 1998 and 2009 were tested for sensitivity to boscalid by determining percentage germination of ascospores and conidia on media amended with boscalid at 0.01 to 10.0 mg/liter. All 31 isolates collected in 1998, 2002, or 2005 were sensitive to boscalid. Of the 170 isolates collected in or after 2006, 84.7% were insensitive to boscalid, including 19 of 30 isolates recovered from greenhouse-grown seedlings. The oldest insensitive isolates were obtained in 2006 from a greenhouse and in 2008 from a commercial field. Ascospores were less sensitive to boscalid than conidia. With boscalid at 1.0 mg/liter, 22.4% of ascospores but only 4.1% of conidia

of 31 sensitive isolates germinated. Similarly, a mean of 68.6% of the ascospores and 54.1% of the conidia of 120 insensitive isolates germinated at 1.0 and 10.0 mg/liter. The 50% effective concentration (EC₅₀) values based on ascospore germination were two to three times higher than values based on conidia germination. The significance of miscalculating EC₅₀ values by considering only conidia was demonstrated in a greenhouse experiment. Twelve isolates that were sensitive, moderately insensitive, or highly insensitive based on conidia germination did not differ in relative virulence on boscalid-treated muskmelon seedlings when inoculum suspensions comprised ascospores alone or ascospores and conidia. This is the first report of differential sensitivity to a fungicide between conidia and ascospores in *D. bryoniae*. Because *D. bryoniae* produces conidia and ascospores on diseased hosts, both spore types should be used when calculating EC₅₀ values for boscalid.

Didymella bryoniae (Fuckel) Rehm (anamorph *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley [5] [synonym *Phoma cucurbitacearum* (Fr.) Sacc.]) is the ascomycete that causes gummy stem blight and black rot of cucurbits. The fungus is homothallic, in that isolates are capable of producing ascospores without pairing with another isolate of a different mating type (9). Although variation among isolates has been observed (9), most isolates have the ability to produce ascospores (26). Pseudothecia with characteristic two-celled ascospores readily form on diseased cucurbit tissues (10,20,45). Ascospores of *D. bryoniae* have been trapped above watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai var. *lanatus*) fields in which the plants had symptoms of gummy stem blight (33). When spores were sampled year-round, ascospores were detected primarily after symptoms of gummy stem blight appeared (34).

The primary method to manage gummy stem blight is protection of cucurbit plants by application of fungicides (16). Strobilurin fungicides, such as azoxystrobin (a methoxy-acrylate) and pyraclostrobin (a methoxy-carbamate), belong to the quinone-outside inhibitor (QoI) cross-resistance group, also known as Fungicide Resistance Action Committee (FRAC) group 11. Their mode of action is binding to ubiquinol oxidase in the electron transport chain in mitochondria, which inhibits fungal respiration (6). Isolates of *D. bryoniae* insensitive to strobilurin fungicides were found in Delaware, Maryland, and Georgia in 2000 (31,39); South Carolina in 2001 (19); and Texas in 2003 (30).

Boscalid is a pyridine-carboxamide, one of the chemical classes in FRAC group 7, which inhibits fungal respiration by binding to the enzyme succinate dehydrogenase in mitochondria (3,4,27). A 2:1 mixture of the fungicides boscalid and pyraclostrobin (Pristine 38WG; BASF Corporation, Research Triangle Park, NC) was registered for use on cucurbits by the United States Environmental Protection Agency in 2003. By 2004, Pristine was applied an average of 1.8 times per year to 25 to 54% of the U.S. watermelon crop (42). In some states, Pristine was recommended as the best fungicide against gummy stem blight (1). The main reason for the widespread use of Pristine was that the boscalid component controlled strobilurin-insensitive isolates of *D. bryoniae* (35). Insensitivity to boscalid in *D. bryoniae* was detected first in Georgia in research plots and commercial fields of watermelon in 2007 (29,38) and subsequently in research plots of watermelon in South Carolina in 2008 (25). Boscalid-insensitive isolates of *D. bryoniae* also have been found in Indiana (12).

In a preliminary experiment with 16 isolates of *D. bryoniae* that included a mix of boscalid-sensitive and -insensitive isolates, it was noted that more ascospores than conidia germinated in the presence of boscalid (25). Because ascospores are airborne but conidia are primarily splash dispersed, a difference in fungicide sensitivity of ascospores versus conidia may have important implications for the epidemiology of gummy stem blight (33,34). The potential differential sensitivity of the two spore types was investigated while a collection of isolates was screened for sensitivity to boscalid. The objectives of this study were to determine (i) if ascospores and conidia of *D. bryoniae* differ in sensitivity to boscalid, (ii) if differential sensitivity of ascospores and conidia affect 50% effective concentration (EC₅₀) calculations for determining sensitivity to boscalid, and (iii) how frequently boscalid-insensitive isolates occur in South Carolina.

Materials and Methods

Isolate collection. In June 2009, four farms producing watermelon were sampled in Bamberg, Barnwell, Colleton, and Hampton Counties, SC. Bamberg, Colleton, and Barnwell Counties were

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among the four South Carolina counties with the largest watermelon acreage in 2007 (43). The field in Bamberg received two applications of Pristine approximately 3 weeks apart. The other three fields also were sprayed with Pristine one or more times during the 2009 growing season. Fifty leaves showing leaf spots of gummy stem blight were randomly selected from one field on each farm. Twenty-five leaves also were collected from a research plot of watermelon at the Clemson University Edisto Research and Education Center, Barnwell County, SC on 25 June. The research plot was included because the detailed fungicide history of the plot was available. In 2009, it received applications of Pristine on 19 May and 5 June.

Isolates of *D. bryoniae* were obtained by culturing from one lesion per leaf. Small pieces of leaf tissue from the margins of lesions were surface disinfested in 0.6% sodium hypochlorite for 1 min, rinsed in sterile deionized water, blotted dry on paper towels, and placed onto one-quarter-strength potato dextrose agar (QPD) amended after autoclaving with 100 mg of chloramphenicol, 100 mg of streptomycin sulfate, and 60.5 mg of metalaxyl (0.25 ml Ridomil 2E) per liter (17). Plates were held at 23 to 25°C with a 16-h photoperiod for 2 to 3 days until pycnidia typical of *D. bryoniae* were produced. In all, 135 single-conidium cultures were stored on dried filter paper at 5°C (28).

In total, 201 isolates were selected for testing in four groups based on their previous field exposure to boscalid-containing fungicide. To determine baseline sensitivity, 15 isolates from a watermelon field in Hampton County collected in May and June 1998 and 6 isolates from muskmelon vines in a field in Florence County, SC, collected in August 2002 were used. These 21 isolates were not previously exposed to strobilurins or boscalid (Table 1) (18,19). Ten isolates collected in July 2005 from two watermelon fields in Barnwell and Hampton Counties represented limited exposure to boscalid for two to three seasons (19). The Barnwell field received three applications of Pristine and the Hampton field received more than one application of Pristine in 2005. In the third group, isolates collected in summer 2009 in South Carolina represented repeated exposure to Pristine. All of the fields sampled in Hampton County in 1998, 2005, and 2009 belonged to the same grower (Table 1). The fourth group included isolates collected after Pristine was registered but it was not known whether the isolates were exposed to boscalid. In 2006, 11 isolates were collected from pollinizer watermelon seedlings with necrotic cotyledons, and 19 isolates were collected in 2009 from watermelon seedlings with symptoms of gummy stem blight (19). In addition, four isolates recovered in May 2008 from watermelon grown in Bamberg County and one isolate recovered from watermelon in July 2008 in

Barnwell County were tested for sensitivity to boscalid; whether they had been exposed to boscalid was unknown.

Boscalid sensitivity. Boscalid (99.5% technical grade) was dissolved in acetone to make a 10 mg/liter stock solution (2). Aliquots of the stock solution were added to autoclaved water agar (20 g of agar per liter) for concentrations of boscalid at 0.01, 0.1, 1.0, and 10 mg/liter, and dispensed into 15-mm petri dishes (2). Control media received only acetone at 1.0 ml/liter. Isolates were grown from a 0.5-by-0.5-cm piece of colonized filter paper onto QPD in petri dishes that were not sealed with Parafilm and held at 23 to 25°C with a 16-h photoperiod for 10 to 14 days (19). Cultures were flooded with 5 ml of sterile water and scraped gently to release conidia and ascospores. The spore suspension was poured through four layers of cheesecloth, and 0.2 ml of the filtrate was spread on boscalid-amended media. Plates were incubated for 24 h at 23 to 25°C with a 16-h photoperiod. At $\times 100$ magnification, 100 conidia and 50 ascospores were examined on each plate for germination. A germinated spore had a germ tube longer than the length of the conidium or one ascospore cell. Spores with germ tubes shorter than the length or no germ tube present at all were counted as nongerminated. Two plates per fungicide concentration were prepared for each isolate, and each isolate was tested twice in separate trials (2). Relative germination was calculated per plate per trial for each isolate and spore type by dividing the number of spores that germinated on fungicide-amended medium by the mean number of spores that germinated on nonamended medium (19). Isolates with an $EC_{50} \leq 1.0$ mg/liter were considered to be sensitive, isolates with an $EC_{50} > 1.0$ and ≤ 10.0 mg/liter were considered moderately insensitive, and isolates with an $EC_{50} > 10.0$ mg/liter were highly insensitive.

Greenhouse experiments. Six isolates collected in 2009 from the farm in Bamberg County and six isolates collected in 2009 from the farm in Barnwell County that differed in boscalid EC_{50} values based on relative conidial germination were used in the greenhouse experiments. Because of space limitations in the greenhouse, the 12 isolates were tested in two groups with 3 isolates from each farm in each group. Within each group, two isolates (one from each farm) were judged to be highly insensitive and the other four isolates were sensitive or moderately insensitive to boscalid in vitro (Table 2). Each group of isolates was tested twice in separate trials. Muskmelon (*Cucumis melo* subsp. *melo* 'Classic') seedlings were transplanted into 10-cm-diameter pots, three uniform seedlings per pot. The experimental design was a split plot, with isolate as the whole plot and fungicide as the subplot, with three replications. Control treatments included noninoculated seedlings treated with fungicides or water, also replicated three times. When seed-

Table 1. The 50% effective concentration (EC_{50}) values calculated by linear regression of probit-transformed relative germination of ascospores, conidia, or both spore types of *Didymella bryoniae* on water agar amended with four concentrations of boscalid

Location ^y	Exposure ^z	Year	N	Spore type	EC_{50} (mg/liter) ^w				Sensitivity ^x		
					Mean $\pm$ SD	Median	Min	Max	S	M	H
Hamp and Flo, fields	None	1998–2002	21	Ascospores	0.26 $\pm$ 0.092	0.25	0.13	0.45	21	0	0
	...			Conidia	0.13 $\pm$ 0.059	0.10	0.057	0.26	21	0	0
	...			Both	0.18 $\pm$ 0.071	0.17	0.09	0.33	21	0	0
Hamp and Barn, fields	Limited	2005	10	Ascospores	0.24 $\pm$ 0.11	0.22	0.12	0.48	10	0	0
	...			Conidia	0.094 $\pm$ 0.078	0.06	0.046	0.28	10	0	0
	...			Both	0.15 $\pm$ 0.090	0.12	0.08	0.37	10	0	0
South Carolina greenhouse	Unknown	2006	11	Ascospores	>10.0	0.23	0.15	>10	7	2	2
	...			Conidia	1.42 $\pm$ 2.70	0.11	0.072	8.17	8	3	0
	...			Both	4.93 $\pm$ 12.0	0.16	0.033	>10	8	2	1
South Carolina greenhouse	Unknown	2009	19	Ascospores	>10.0	>10.0	0.20	>10	3	3	13
	...			Conidia	>10.0	>10.0	0.12	>10	4	4	11
	...			Both	>10.0	>10.0	0.16	>10	3	4	12
Hamp, Bam, Barn, Coll; fields	Repeated	2009	37	Ascospores	4.71 $\pm$ 2.72	4.43	0.82	10.24	3	32	2
	...			Conidia	1.08 $\pm$ 0.84	0.86	0.18	4.58	23	14	0
	...			Both	2.13 $\pm$ 1.29	1.95	0.39	5.33	9	28	0

^wSD = standard deviation, Min = minimum, and Max = maximum.

^xSensitive (S), $EC_{50} \leq 1.0$ mg/liter; moderately insensitive (M), $EC_{50} > 1.0$ mg/liter and ≤ 10.0 ; and highly insensitive (H), $EC_{50} > 10.0$ mg/liter.

^y Hampton (Hamp), Florence (Flo), Barnwell (Barn), Bamberg (Bam), and Colleton (Coll) Counties in South Carolina; greenhouse or field.

^zExposure to boscalid.

lings had two fully expanded true leaves, seedlings were sprayed with boscalid (Endura) at 0.28 g/liter, pyraclostrobin (Cabrio 20EG) at 0.14 g/liter, boscalid + pyraclostrobin (Pristine 38WG) at 0.43 g/liter, or water with a handheld pump-pressurized sprayer. The rate of boscalid + pyraclostrobin was equivalent to the labeled rate per 935 liters of water applied per hectare, and the rates of boscalid and pyraclostrobin were equivalent to the amounts of these two fungicides in Pristine.

One day later, seedlings were inoculated with a suspension of 1 to 2×10^6 ascospores and conidia per milliliter of sterile sucrose-casein solution (7). Inoculum consisted of only ascospores for three isolates and a mix of ascospores and conidia for nine isolates. Ascospores represented $\geq 65\%$ of the spores in the suspensions for eight of the nine isolates (Table 2). Inoculum was applied with a handheld pump-pressurized sprayer that delivered approximately 1 ml per seedling. Noninoculated control seedlings were sprayed with sterile sucrose-casein solution. Seedlings were held at 100% relative humidity for 72 h. Three days after inoculation, percentage of diseased foliage for each plant was rated visually by assigning a rating from a 16-point Horsfall-Barratt-type rating scale, where 0 = 0, 7 = 45, and 15 = 100% (22). The midpoint percentage of each rating was used for data analysis. A percent mean disease severity for each pot (experimental unit) was calculated. To account for differences in virulence among individual isolates, relative disease severity was calculated as the percent disease severity on fungicide-treated seedlings divided by the percent disease severity on the inoculated water control (19). Disease severity was used to compare fungicide treatments with the nontreated control, and relative disease severity was analyzed to compare fungicides after correcting for possible differences in virulence among isolates (26).

Statistical analysis. All spore germination data sets exhibited equality of variances between the two repeated tests based on two-tailed *F* tests of mean square error terms ($P = 0.025$); therefore, data were combined for analysis (37). Relative germination data were transformed with the arcsine square root before analysis of variance to reduce inequality of variances among treatments. PROC MIXED of SAS (version 9.1; SAS, Inc., Cary, NC) was used to examine the effects of spore type and isolate as fixed effects and replication nested within trial (i.e., repetition of an experiment) as a random effect. When relative spore germination was plotted against the base-10 logarithm of boscalid concentration, plots for most isolates were not linear. Relative spore germination was probit transformed and regressed against the base-10 loga-

rithm of the boscalid concentration using PROC GLM of SAS (19,39). The resulting linear regression equations were solved to calculate the concentration of boscalid at which the relative responses were reduced to 50% of the value of the nonamended control (EC_{50}). Because of significantly higher germination of ascospores compared with conidia on boscalid-amended medium, germination data from ascospores, conidia, and both spore types were used to calculate EC_{50} values. With greenhouse data, disease severity and relative disease severity percentages were transformed by calculating the arcsine square root before analysis of variance to reduce inequality of variances among treatments. PROC MIXED of SAS was used to examine the effects of trial, isolate, fungicide, and their interactions as fixed effects and replication nested within trial-isolate as a random effect (19).

Results

Spore germination on boscalid-amended medium. All 21 baseline isolates, which were collected in 1998 from watermelon or in 2002 from muskmelon, were sensitive to boscalid. A mean of 99.8% of the ascospores and 99.3% of conidia germinated on unamended water agar. Except for a single ascospore of isolate W277 which was collected in 1998, no other ascospores and no conidia of baseline isolates germinated on water agar amended with boscalid at 10.0 mg/liter (*data not shown*). Conidia of five isolates did not germinate with boscalid at 1.0 mg/liter. Conidia of 10 isolates and ascospores of 20 isolates germinated at 1.0 mg/liter (*t* tests of least-square means compared with 0, $P \leq 0.05$). There was no interaction between spore type and isolate ($P = 0.72$). Mean relative germination percentages of 25.2% for ascospores and 5.2% for conidia differed significantly ($P < 0.0001$).

Mean germination of ascospores and conidia of 10 isolates collected in 2005 that had been exposed to boscalid for ≤ 3 years was 99.7 and 99.0%, respectively, on unamended water agar. All 10 isolates were sensitive to boscalid. Conidia of three isolates did not germinate with boscalid at 1.0 mg/liter but ascospores of all isolates did. Relative germination of conidia of four other isolates was $\leq 1.3\%$ and not significantly greater than 0 (*t* tests of least-square means, $P = 0.05$). Although there was an interaction between spore type and isolate with boscalid at 1.0 mg/liter ($P = 0.008$), pairwise comparisons of relative germination between ascospores and conidia differed for all 10 isolates ($P \leq 0.04$). Mean relative germination percentages of 16.9% for ascospores and 2.2% for conidia differed significantly ($P < 0.0001$). No spores germinated on water agar amended with boscalid at 10.0 mg/liter.

Table 2. Comparison of virulence of 12 isolates of *Didymella bryoniae* that differed in 50% effective concentration (EC_{50}) values for boscalid and were inoculated onto muskmelon seedlings treated with boscalid, pyraclostrobin, pyraclostrobin + boscalid, or water in a greenhouse^x

Isolate	County	Boscalid EC ₅₀ (mg/liter)			Inoculum (10 ⁶ /ml)		DS (%) ^y	RDS (%) ^z
		Con	Asco	Con + Asco	Con	Asco		
Group 1								
W681	Barnwell	0.19	3.20	0.61	0.4	1.6	11.0 b	85.0 a
W711	Barnwell	0.39	5.87	1.37	0.2	1.8	12.9 b	60.0 a
W709	Barnwell	>10.00	>10.00	>10.00	0.4	1.6	9.2 b	42.2 a
W771	Bamberg	1.21	5.51	2.41	0.2	1.8	39.7 ab	75.5 a
W764	Bamberg	1.61	9.19	4.08	0.2	1.6	56.1 a	69.5 a
W766	Bamberg	>10.00	>10.00	>10.00	0.7	1.3	29.2 ab	57.5 a
Group2								
W678	Barnwell	0.31	1.38	0.61	0.0	1.0	18.6 a	76.0 a
W708	Barnwell	0.74	>10.00	2.65	0.0	1.5	14.8 a	69.6 a
W686	Barnwell	>10.00	>10.00	>10.00	0.3	1.5	29.5 a	72.7 a
W798	Bamberg	0.12	2.50	0.62	0.0	1.5	11.3 a	68.6 a
W773	Bamberg	4.58	6.09	5.33	0.2	1.7	9.2 a	64.0 a
W800	Bamberg	>10.00	>10.00	>10.00	0.4	1.1	10.5 a	73.3 a

^x Con = conidia, Asco = ascospores. Means within groups followed by the same letter are not significantly different (*t* tests, $P = 0.01$).

^y Disease severity (DS). Mean across two trials, four fungicide treatments (three fungicides and the water control), three replications, and three seedlings rated individually per replication. Values shown are back-transformed least-square means from arcsin-square root transformed values used in analysis of variance.

^z Relative disease severity (RDS) = percentage disease severity on fungicide-treated seedlings divided by the percentage disease severity on the water control. Means were calculated across two trials, three fungicides, three replications, and three seedlings rated individually per replication. Values shown are back-transformed least-square means from arcsin-square root transformed values used in analysis of variance.

Varying percentages of ascospores and conidia of 37 isolates collected in 2009 from watermelon sprayed with Pristine germinated on water agar amended with boscalid at 10 mg/liter (Table 1). Mean germination of ascospores and conidia on unamended water agar was 99.9 and 98.3%, respectively. With boscalid at 10.0 mg/liter, there was no interaction between spore type and isolate ($P = 0.96$). Mean relative germination percentage at 10.0 mg/liter was higher for ascospores (29.0%) than for conidia (11.7%) ($P < 0.0001$). Relative germination percentage for conidia of five isolates was not significantly greater than 0, whereas the relative germination percentage for ascospores for all isolates was greater than 0 (t tests of least-square means, $P = 0.05$).

EC₅₀ values for boscalid. Because ascospores consistently germinated more frequently than conidia on boscalid-amended media, it was not clear which spore type should be used to calculate EC₅₀ values. Therefore, EC₅₀ values were calculated with relative germination of conidia and ascospores separately and with mean relative germination across both spore types on media amended with four concentrations of boscalid. Isolates with an EC₅₀ $\leq$ 1.0 mg/liter were considered to be sensitive, because no conidia and only 1 ascospore of 1 of 31 baseline isolates germinated with boscalid at 10.0 mg/liter. Isolates with an EC₅₀ $>$ 1.0 and $\leq$ 10.0 mg/liter were considered moderately insensitive, and isolates with an EC₅₀ $>$ 10.0 were highly insensitive. At least 3% of conidia and 13% of ascospores of all insensitive isolates germinated with boscalid at 10.0 mg/liter (*data not shown*). Mean and median EC₅₀ values and the range of values for the 68 isolates discussed above (the three groups of isolates that were not exposed, minimally exposed, or repeatedly exposed) were largest with ascospores, intermediate with both spore types, and lowest with conidia (Table 1). All 31 isolates collected prior to or in 2005 had EC₅₀ values $<$ 1.0 based on germination of ascospores, conidia, and both spore types and were sensitive to boscalid.

The most variation among spore types was observed with 37 of the 135 isolates collected in 2009 from South Carolina watermelon fields. The EC₅₀ values of these 37 isolates were $\leq$ 10 mg/liter but still within the range of boscalid concentrations used. Based on conidia germination, 23 (62%) of these isolates were judged to be sensitive to boscalid, with an EC₅₀ $\leq$ 1.0 mg/liter. However, with ascospore germination and with mean germination across conidia and ascospores, $<$ 25% of the isolates were classified as sensitive and $\geq$ 75% of the isolates were considered insensitive (Table 1).

With another group of 68 insensitive isolates selected randomly from the remaining 98 (of 135) 2009 isolates, 1 (1.5%), 30 (44%), and 14 (21%) isolates had EC₅₀ values $>$ 1.0 and $\leq$ 10 mg/liter based on germination of ascospores, conidia, and both spore types, respectively. Of these 68 isolates, 38 were classified as highly insensitive, with EC₅₀ values $>$ 10.0 based on conidia germination. These isolates also were highly insensitive based on germination of ascospores and both spore types, except one isolate that had an EC₅₀ value of 9.96 based on ascospore germination.

Greenhouse experiments. All 12 isolates of *D. bryoniae* collected from Bamberg or Barnwell counties in 2009 and tested in

the greenhouse caused gummy stem blight on muskmelon seedlings previously treated with water, boscalid, pyraclostrobin, or boscalid + pyraclostrobin (Table 2). No symptoms developed on any seedlings in any trial that were sprayed with water and then with sucrose-casein. One noninoculated control seedling in one pot sprayed with boscalid + pyraclostrobin in the second trial had one small leaf spot from which *D. bryoniae* was recovered. Disease severity on inoculated seedlings sprayed with water ranged from 17 to 72% for the 12 isolates. Levels of disease severity in the four fungicide treatments were not affected by differing amounts of ascospores or conidia in the inoculum suspensions, based on correlation analysis (*data not shown*).

Isolates were selected for greenhouse testing because their EC₅₀ values based on conidial germination indicated they were sensitive, moderately insensitive, or highly insensitive to boscalid in vitro. With both groups of isolates, there was a highly significant effect of fungicide treatment on the severity of gummy stem blight (Table 3). In trial 1 with the first group of isolates and in trial 2 with the second group of isolates, all three fungicides reduced severity of gummy stem blight compared with the water control but did not differ from each other (Fig. 1). With the first group of isolates in trial 2, boscalid + pyraclostrobin had less gummy stem blight than the water control and boscalid (Fig. 1). With the second group of isolates, fungicides did not differ from the water control in trial 1 (Fig. 1). Disease severity varied among isolates in the first group, whereas isolates in the second group did not differ from each other (Tables 2 and 3). With both groups of isolates, there was no isolate–fungicide interaction, which indicated that the response of all isolates to fungicide treatments was similar, despite the expected

Fig. 1. Mean severity of gummy stem blight on muskmelon seedlings treated with fungicides the day before inoculation with 1 of 12 isolates of *Didymella bryoniae*. The trial–fungicide interaction was significant for group 2. Means within groups and trials followed by the same letter are not significantly different (t tests, $P = 0.01$).

Table 3. Analysis of variance with severity of gummy stem blight caused by 12 isolates of *Didymella bryoniae* on muskmelon seedlings treated with pyraclostrobin, boscalid, pyraclostrobin + boscalid, or water in a greenhouse^a

Source of variation	Degrees of freedom		Group 1		Group 2	
	Numerator	Denominator	F	Prob > F	F	Prob > F
Trial	1	21	0.43	0.52	1.60	0.22
Isolate	5	21	4.28	0.0077	0.70	0.63
Trial × isolate	5	21	0.57	0.72	1.94	0.13
Fungicide	3	63	17.68	<0.0001	9.09	<0.0001
Trial × fungicide	3	63	0.38	0.76	10.34	<0.0001
Isolate × fungicide	15	63	0.65	0.82	0.91	0.56
Trial × isolate × fungicide	15	63	1.40	0.17	1.04	0.43

^a Six isolates, three from one farm in Bamberg County and one farm in Barnwell County, SC, that differed in preliminary 50% effective concentration (EC₅₀) values for boscalid based on conidial germination on boscalid-amended media were included in each group. Within each group, two isolates (one from each farm) were judged to be highly insensitive and the other four isolates were sensitive or moderately insensitive to boscalid in vitro. F = F value and Prob = probability.

differential response to boscalid, based on conidia germination in vitro (Table 3).

Relative disease severity was calculated for each isolate to assess disease development on fungicide-treated plants relative to the water control, as was done with spore germination in vitro (19). With both groups of isolates, there were no significant differences between the two trials, no significant differences among the six isolates within a group, and no significant interactions, including no isolate–fungicide interaction (Table 4). The lack of an isolate–fungicide interaction and the lack of a significant isolate effect after differences in virulence among isolates were accounted for indicated that the response of all isolates to the three fungicides was similar (Table 2). Mean relative disease severity for each isolate was significantly greater than 0 with each of the three fungicides (t tests, $P \leq 0.002$). Thus, the three fungicides did not suppress gummy stem blight development by any of the 12 isolates.

With the first group of isolates, effects of treatment were significant (Table 4) because relative disease severity was greater on boscalid-treated plants (74.8%) than on plants treated with boscalid + pyraclostrobin (54.7%) (t tests of least-squares means, $P = 0.01$). Mean relative disease severity on pyraclostrobin-treated plants was intermediate (66.7%) and did not differ from the values for the other two fungicides. With the second group of isolates, relative disease severity averaged across all treatments was higher in trial 1 (81%) than in trial 2 (51%) (Table 4) but there were no significant interactions of treatment–trial. Mean relative disease severity on seedlings treated with boscalid, boscalid + pyraclostrobin, or pyraclostrobin was 62.4, 76.0, and 73.4%, respectively, of disease severity on the water control (set to 100%) and did not differ among fungicides.

Sensitivity of isolates from South Carolina to boscalid. Based on the results of in vitro and greenhouse experiments, sensitivity to boscalid was evaluated based on EC_{50} values calculated with mean relative germination of ascospores and conidia together. Isolates with EC_{50} values > 1.0 mg/liter were considered insensitive to boscalid. All five isolates of *D. bryoniae* collected in two watermelon fields in 2008 were highly insensitive to boscalid with EC_{50} values > 10 mg/liter. Of the 135 field isolates collected in 2009, 15 (11%) were sensitive (i.e., had EC_{50} values < 1.0 mg/liter) and the remaining 120 (89%) were insensitive. Ascospores and conidia of these sensitive isolates that had been exposed to boscalid had higher relative germination (64.1 and 45.4%, respectively) than baseline isolates at 1.0 mg/liter. At 10.0 mg/liter, relative germination averaged 14.0% for ascospores and 4.4% for conidia. Of the insensitive isolates, 54 isolates were moderately insensitive (i.e., had EC_{50} values > 1.0 mg/liter and < 10.0 mg/liter) and 66 isolates (49% of the total and 55% of the insensitive isolates) were highly insensitive, with EC_{50} values > 10.0 mg/liter. The frequency distributions of isolates on three farms in Hampton, Bamberg, and Barnwell Counties were similar, and a majority of the isolates were highly insensitive (Fig. 2). On the farm in Colleton County, from which only six isolates were recovered, half of the isolates were

sensitive. In the research plots in which Pristine had been used in rotation with other fungicides, the majority of the isolates were moderately insensitive. Overall, 92.6% of the South Carolina isolates collected in watermelon fields in 2008 and 2009 were insensitive. Insensitive isolates also were found in 2006 and 2009 in collections of isolates from the same greenhouse in South Carolina. In the 2006 collection of 11 isolates, 3 were insensitive and, in the 2009 collection of 19 isolates, 16 were insensitive (Table 1). Thus, 63% of the isolates from the greenhouse were insensitive. Of the 170 isolates collected in South Carolina since 2006, 84.7% (144 isolates) were insensitive to boscalid.

Discussion

This is the first report of differential sensitivity to a fungicide between spore types in *D. bryoniae*. Ascospores of *D. bryoniae* germinated more frequently (1.6 to 8.4 times) on boscalid-amended media than conidia did. Differences in sensitivity of ascospores and conidia of *D. bryoniae* to boscalid were apparent in baseline isolates and in exposed isolates that were sensitive, moderately insensitive, and highly insensitive. Ascospores were not examined in other previously published boscalid-sensitivity studies with *D. bryoniae* or the related species, *D. rabiei* (38,39,46). Ascospores and conidia of *Leptosphaeria maculans*, another ascomycete pathogen in the family Pleosporales, also responded differently to fungicides (11). Ascospores of *L. maculans* were insensitive to low concentrations (≤ 4 μ g/ml) of flusilazole, tebuconazole, and benomyl, whereas conidia were sensitive. At least 98% of both ascospores and conidia of *D. bryoniae* had

Fig. 2. Frequency distribution of boscalid 50% effective concentration (EC_{50}) values calculated from germination of ascospores and conidia of 135 isolates of *Didymella bryoniae* collected from watermelon on four farms in Bamberg (Bg), Barnwell (Bw), Colleton (C), and Hampton (H) Counties, SC, and from research plots (EREC) in Barnwell County, SC, in 2009.

Table 4. Analysis of variance for relative disease severity of 12 isolates of *Didymella bryoniae* inoculated on muskmelon seedlings treated with pyraclostrobin, boscalid or pyraclostrobin + boscalid in a greenhouse^z

Source of variation	Degrees of freedom		Group 1		Group 2	
	Numerator	Denominator	F	Prob > F	F	Prob > F
Trial	1	18	0.06	0.80	12.24	0.0026
Isolate	5	18	1.22	0.34	0.10	0.99
Trial × isolate	5	18	2.46	0.07	1.48	0.24
Fungicide	2	36	3.55	0.04	1.30	0.29
Trial × fungicide	2	36	0.20	0.82	1.08	0.35
Isolate × fungicide	10	36	0.84	0.59	1.29	0.27
Trial × isolate × fungicide	10	36	1.82	0.09	1.20	0.32

^z Relative disease severity = percent disease severity on fungicide-treated seedlings divided by the percent disease severity on the water control. Six isolates, three from one farm in Barnwell County and one farm in Barnwell County, SC, that differed in preliminary 50% effective concentration (EC_{50}) values for boscalid based on conidial germination on boscalid-amended media were included in each group. Within each group, two isolates (one from each farm) were judged to be highly insensitive and the other four isolates were sensitive or moderately insensitive to boscalid in vitro. F = F value and Prob = probability.

germinated after 24 h on unamended water agar. Although ascospores of *D. rabiei* germinated more rapidly than conidia in another study, germination of both spore types on water agar reached a maximum after 12 to 24 h (41). Thus, it was very unlikely that the differences observed in sensitivity to boscalid between ascospores and conidia of *D. bryoniae* at 24 h were due to differences in germination rates. Whether differential sensitivity to boscalid is related to ascospores being two-celled is unknown. Fungicides decreased the number of germ tubes produced by *L. maculans* from terminal cells of the multicellular ascospores but not from interstitial cells (11). Sensitivity of ascospores and conidia of *D. bryoniae* to fungicides other than boscalid is being tested in additional experiments.

The potential exists for short-distance dispersal of fungicide-insensitive isolates of *D. bryoniae* via ascospore transport. Ascospores of *Mycosphaerella graminicola*, an ascomycete pathogen of wheat, that carried the strobilurin-insensitivity mutation G143A were detected up to 85 m from an infected crop treated with trifloxystrobin (14). All isolates of *D. bryoniae* used in this study readily formed ascospores and conidia on QPDA when cultured as described herein. In the field, *D. bryoniae* produced pseudothecia, asci, or ascospores on vegetative parts of 18 different cultivars of nine cultivated cucurbits (20). In another study, pseudothecia formed on overwintered muskmelon debris (18). Ascospores also were produced on cucumber debris inside and outside greenhouses (44). Ascospores of *D. bryoniae* were trapped in the air 1.2 m above a field of watermelon with gummy stem blight in Florida (34). How far ascospores of *D. bryoniae* are dispersed away from infected plants has not been determined.

The hypothesis tested in the greenhouse was that isolates of *D. bryoniae* with low EC₅₀ values (based on conidial germination on boscalid-amended media) would show a different response on plants treated with boscalid than isolates with high EC₅₀ values (i.e., they would have lower gummy stem blight severity ratings). Because there was no isolate–fungicide interaction for either percent severity or relative severity (adjusted for differences in virulence of isolates), all 12 isolates responded similarly to the fungicides regardless of EC₅₀. Because more ascospores than conidia germinated when exposed to boscalid in vitro, the presence of ascospores in the inoculum suspensions of all isolates likely resulted in a substantial percentage of germinated spores and significant development of gummy stem blight on boscalid-treated seedlings. Isolates appeared to be more sensitive to boscalid in vitro than they were in the greenhouse, because fewer conidia than ascospores germinated on boscalid-amended media. Thus, calculating EC₅₀ values based on conidia alone underestimated isolates' actual insensitivity to boscalid. This miscalculation had the largest impact on EC₅₀ values for moderately insensitive isolates, which are the isolates of primary interest when insensitivity is first detected. Because *D. bryoniae* produces two types of spores in the field, both ascospores and conidia should be used when calculating EC₅₀ values for boscalid.

The percentage of field isolates of *D. bryoniae* that were insensitive to boscalid was relatively similar in South Carolina (92%) and Georgia (83.5%) (40). The percentage may have been lower in Georgia due to different methods, because a single concentration (3 mg/liter) and a mycelia growth assay were used. Baseline isolates from Georgia assayed with mycelia growth had a median EC₅₀ of 0.055 µg/ml, which also was lower than the values obtained with spore germination in the present study (38). Baseline EC₅₀ values based on germination of conidia of *D. rabiei* ranged from 0.018 to 0.50 µg/ml, with a mean of 0.19 µg/ml (46). This mean was slightly higher than the mean of 0.13 µg/ml based on conidia germination by *D. bryoniae* in the present study but still fell within the same order of magnitude.

In the greenhouse experiment, severity of gummy stem blight caused by one group of isolates was somewhat lower on plants treated with Pristine than on plants treated with either Cabrio or Endura; with the other group of isolates, there were no differences among the three fungicides. In a field naturally infested with stro-

bilurin- and boscalid-insensitive isolates of *D. bryoniae*, gummy stem blight was severe and progressed similarly on watermelon plants treated with boscalid, pyraclostrobin, or boscalid + pyraclostrobin (21). Thus, Pristine may provide better control of gummy stem blight than boscalid or pyraclostrobin with some doubly insensitive isolates but this effect appears to be isolate dependent. However, the level of control observed in the greenhouse and field was not enough to prevent substantial yield loss if disease started during a critical period for fruit production (16).

Strobilurin-insensitive isolates of *D. bryoniae* previously were found in watermelon transplant-production greenhouses in Georgia (39) and South Carolina (19). Thus, there is a risk of spreading fungicide-insensitive isolates on transplants if transplants with symptoms of gummy stem blight are shipped to growers. The pollenizer watermelon seedlings from which the boscalid-insensitive isolates were obtained in 2006 were not distributed to growers because they were too severely diseased. The three farms in Bamberg, Barnwell, and Hampton Counties sampled in 2009 all obtained watermelon transplants from the greenhouse sampled in 2009. It is not known whether any of the transplants supplied to the three farms in 2009 had symptoms of gummy stem blight on them. It is interesting that the frequency distributions of EC₅₀ values on the three farms were much more similar to each other than to the distributions on the farm in Colleton and the research plots, both of which were transplanted with seedlings from other sources. However, insensitive isolates of *D. bryoniae* were present on other farms in Bamberg and Barnwell Counties since 2008, and insensitive isolates also were found on the farm in Colleton and in the research plots. Thus, the role of transplants in dissemination of boscalid-insensitive isolates remains unclear.

Mixing fungicides with two different modes of action has been widely recommended as a strategy to prevent development of insensitivity to fungicides with site-specific modes of action, such as strobilurins and boscalid (8,36). Strobilurin-insensitive isolates of *D. bryoniae* were present in Georgia and South Carolina 3 and 2 years, respectively, before Pristine was registered in 2003 (19,39). Applying Pristine to a strobilurin-insensitive population should have been equivalent to applying boscalid alone. If watermelon growers in Georgia and South Carolina began using Pristine in 2003 when it was registered, then insensitivity to boscalid developed after 5 and 6 years of use, respectively (25,38). This period of time is twice as long as the period from the registration of strobilurins as “stand-alone” products on cucurbits in 1998 to the first detection of strobilurin-insensitive isolates of *D. bryoniae* in 2000 (19,39). It appears that the mixture of boscalid with pyraclostrobin may have slowed development of insensitivity to boscalid in *D. bryoniae*, possibly due to uneven distribution of strobilurin-insensitive isolates or the occurrence of mixed populations of sensitive and insensitive isolates (19,39).

A new member of FRAC group 7 (the succinate-dehydrogenase inhibitors) is fluopyram (Luna SC500; Bayer CropScience, Research Triangle Park, NC), a pyridinyl-ethyl-benzamide. Fluopyram apparently binds to a different target site in ascomycete fungi than boscalid does, because it was effective against boscalid-insensitive isolates of three cucurbit pathogens, including *D. bryoniae* (15,24). Formulations of fluopyram to be registered on cucurbits to control gummy stem blight and other diseases will include either trifloxystrobin or tebuconazole (13). Because boscalid insensitivity appeared after insensitivity had developed to its formulation partner, pyraclostrobin, combining fluopyram with trifloxystrobin might not prevent insensitivity to fluopyram, because cross-resistance exists among strobilurin fungicides (47). Mixing fluopyram with tebuconazole, however, should be an effective strategy to delay or prevent insensitivity, because tebuconazole is effective against isolates of *D. bryoniae* that are insensitive to boscalid and pyraclostrobin (23,32).

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