Resistance to Pyraclostrobin and Boscalid in Populations of *Botrytis cinerea* from Stored Apples in Washington State

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ABSTRACT

Kim, Y. K., and Xiao, C. L. 2010. Resistance to pyraclostrobin and boscalid in populations of *Botrytis cinerea* from stored apples in Washington State. Plant Dis. 94:604-612.

Gray mold caused by *Botrytis cinerea* is a major postharvest disease of apple. Pristine, a formulated mixture of pyraclostrobin and boscalid, was recently registered for use on apple. Pristine applied within 2 weeks before harvest is effective in controlling gray mold in stored apple fruit. To determine the baseline sensitivity of *B. cinerea* populations to these fungicides, 40 isolates from organic and 80 from conventional apple orchards where Pristine had not been used were tested for mycelial growth or conidial germination on fungicide-amended media. To monitor fungicide resistance, gray-mold-decayed apple fruit originating from orchards in which Pristine had been used were sampled from a fruit packinghouse. Isolates of *B. cinerea* recovered from the fruit were tested for resistance to the two fungicides. In the in vivo study in the orchards, Pristine was applied to fruit 1 day before harvest. Fruit were then harvested, wounded, and inoculated with isolates exhibiting different fungicide-resistance phenotypes. Fruit were stored at 0°C for 8 weeks for decay development. The effective concentration that inhibits mycelial growth by 50% relative to the control (EC₅₀) values for sensitive isolates ranged from 0.008 to 0.132 µg/ml (mean = 0.043, *n* = 116) for pyraclostrobin and from 0.003 to 0.183 µg/ml (mean = 0.075, *n* = 117) for Pristine in a mycelial growth assay on potato dextrose agar. The EC₅₀ values of boscalid for sensitive isolates ranged from 0.065 to 1.538 µg/ml (mean = 0.631, *n* = 29) in a conidial germination assay on water agar. Four isolates were resistant to pyraclostrobin, with resistance factors (RFs) ranging from 12 to 4,193. Of the four pyraclostrobin-resistant isolates, one also was resistant to boscalid (RF = 14) and Pristine (RF = 373), and two exhibited reduced sensitivity to Pristine (RF = 16 and 17). The minimum inhibitory concentration for conidial germination (for boscalid) or mycelial growth (for pyraclostrobin and Pristine) of sensitive isolates was 5 µg/ml, which is thus recommended as a discriminatory concentration for phenotyping isolates for resistance to these fungicides. Of the 56 isolates obtained from decayed apple fruit that had been exposed to Pristine, 11 (approximately 20%) were resistant to both pyraclostrobin and boscalid and 1 was resistant only to pyraclostrobin. Of the additional 43 isolates obtained from decayed apple fruit originating from an organic orchard, 3 were resistant only to pyraclostrobin, 2 were resistant only to boscalid, and 2 were resistant to both fungicides. It appeared that there was no cross resistance between pyraclostrobin and boscalid because of the existence of isolates resistant only to either pyraclostrobin or boscalid. Pristine applied at label rate in the orchard failed to control gray mold on apple fruit inoculated with the Pristine-resistant isolates. This is the first report of multiple resistance to pyraclostrobin, boscalid, and Pristine in field populations of *B. cinerea*. Our results suggest that the development of dual resistance to pyraclostrobin and boscalid in *B. cinerea* populations could result in the failure to control gray mold with Pristine.

Botrytis cinerea Pers.:Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel), one of the most important plant-pathogenic fungi, is responsible for gray mold diseases on more than 230 plant species worldwide (14). Substantial economic losses resulting from diseases caused by *B. cinerea* occur not only during the crop growing season but also after harvest, during storage and transit of agricultural products (6). Control of gray mold dis-

eases caused by *B. cinerea* is largely dependent on the use of synthetic fungicides (19). However, *B. cinerea* is a high-risk fungal pathogen in the development of resistance to fungicides because of its high genetic variability, short life cycle, and prolific reproduction (5,28). Resistance of *B. cinerea* to fungicides can often result in the failure of disease control (19).

Gray mold caused by *B. cinerea* is a common postharvest fruit rot disease of apple worldwide. In a survey conducted in Washington State during 2003–05, gray mold accounted for approximately 28% of the total fruit rots on apple fruit during storage (17). Conidia of *B. cinerea* produced on infected plant materials and on germinating sclerotia are the sources of inoculum in the orchard. Conidia may also be brought into packinghouse flume and

dump tank water through contaminated bins and fruit. Punctures, bruises, and insect damage created near or at harvest and during postharvest handling are the primary avenue for *B. cinerea* to infect fruit (27). Although *B. cinerea* can also infect floral parts of the fruit during bloom in the orchard, leading to calyx-end gray mold (18), this type of gray mold resulting from quiescent infections is not commonly seen on fruit grown in the U.S. Pacific Northwest (18). Protecting wounds from infection by *B. cinerea* is essential to gray mold control in apple.

A postharvest fungicide in combination with diphenylamine (DPA; an antioxidant) as a drench treatment may be applied to apple fruit prior to storage for control of postharvest diseases and superficial scald (browning of apple fruit peel, a physiological disorder). Thiabendazole (TBZ) was the most commonly used postharvest fungicide on apple fruit prior to the registration of fludioxonil and pyrimethanil in 2004 for postharvest use on pome fruit in the United States. TBZ is still effective for controlling gray mold in apple fruit because the vast majority of *B. cinerea* isolates from apple fruit in Washington State are sensitive to TBZ (36); however, resistance to TBZ is common in *Penicillium expansum* and often results in the failure of blue mold control (20). Fludioxonil and pyrimethanil applied as a prestorage drench treatment are highly effective in controlling gray mold in apple (30,36). However, there are concerns about potential contamination of apple fruit by food-borne pathogens during postharvest drenching as well as the build-up of apple pathogens in the recirculating drench solution. Alternatives to DPA drench include treating closed storage rooms with 1-methycyclopropene (1-MCP) as a vapor treatment or micronized DPA as a fog treatment (4,8). Currently 1-MCP is widely used to treat apple fruit for retaining postharvest fruit quality and control of scald (7,8). As a potential alternative to postharvest fungicide drenches for control of postharvest diseases on the apple fruit that are destined for subject to treatments with 1-MCP or micronized DPA, a preharvest spray with Pristine (a premixed formulation of pyraclostrobin and boscalid) applied within 2 weeks before harvest has been shown effective in controlling gray mold (34). Pristine was registered in 2005 for use on apple in the United States. Cur-

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Accepted for publication 3 February 2010.

doi:10.1094/PDIS-94-5-0604
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rently, preharvest application of Pristine for postharvest disease control on apple is being increasingly implemented by apple growers and packers in the Pacific Northwest.

Pyraclostrobin is a strobilurin fungicide, belonging to the group of quinone outside inhibitor (QoI) fungicides. QoI fungicides inhibit mitochondrial respiration by binding at the Qo site of the cytochrome b and, thus, block electron transfer between cytochrome b and cytochrome c₁, resulting in a disruption of the energy cycle of the fungus (3). Because of their broad-spectrum activity against major groups of plant-pathogenic fungi, effectiveness in controlling fungal isolates resistant to other mode-of-action fungicides, low use rates, and some yield benefits, QoIs have become one of the most important groups of fungicides in agriculture (3). Previous studies showed that QoI fungicides have a protective, curative, and eradicated effect by inhibiting spore germination, mycelial growth, and sporulation of the fungal pathogens; however, individual QoI fungicides may vary in their levels of activity against specific pathogens and diseases (3). Because of their site-specific mode of action, risk for development of resistance to QoIs is considered high (9,21). Numerous fungal pathogens from various crops, including *B. cinerea*, have developed resistance to QoI fungicides (10,13,15).

Boscalid belongs to the carboxamide class of fungicides, with a different mode-of-action from QoI fungicides, though both classes are respiration-inhibitor fungicides. Boscalid prevents mitochondrial respiration by inhibiting the activity of mitochondrial respiratory complex II, which is also referred to as succinate dehydrogenase (SDH). SDH is a key enzyme in the tricarboxylic acid cycle and catalyzes the coupled reaction of the oxidation of succinate to fumarate and the reduction of ubiquinone to ubiquinol (29,32). Boscalid is effective against spore germination and mycelial growth and has a broad-spectrum activity against a wide range of important plant-pathogenic fungi such as *Alternaria* spp., *Botrytis* spp., *Sclerotinia* spp., *Mycosphaerella* spp., *Monilinia* spp., and powdery mildews (32). Although SDH inhibitor fungicides are considered medium-risk for the development of resistance in fungal pathogens (9), resistance to boscalid has been found in field isolates of several fungal pathogens, including *Alternaria alternata* (1), *Corynespora cassiicola* (23), *Didymella bryoniae* (16), and *Podosphaera xanthii* (22). Resistance to boscalid has also been observed in field isolates of *B. cinerea* in Europe but no detailed information, such as frequency of resistant isolates and range of EC₅₀ values for resistant isolates, was available (32). No dual resistance to pyraclostrobin and boscalid has been reported in field isolates of *B. cinerea*.

The objectives of this study were to (i) establish the distribution of baseline sensitivity to pyraclostrobin, boscalid, and Pristine in field populations of *B. cinerea* from apple; (ii) determine whether resistance to pyraclostrobin and boscalid occurred in field isolates of *B. cinerea* from apple orchards where Pristine had been used; and (iii) determine whether the effectiveness of Pristine was compromised for control of gray mold incited by pyraclostrobin- and boscalid-resistant isolates of *B. cinerea*.

MATERIALS AND METHODS

Fungal isolates. In total, 120 single-spore isolates of *B. cinerea* were included in the study for establishing baseline sensitivity. Of the 120 isolates, 80 were recovered during 2001–03 from either decayed apple fruit sampled from fruit packing-houses or floral parts of apple fruit sampled from commercial apple orchards in the Wenatchee, Columbia Basin, Okanogan, and Yakima areas in central Washington, and 40 were obtained in 2007 and 2008 from decayed apple fruit that originated from commercial organic orchards in the Okanogan and the Columbia Basin areas. These isolates were collected either prior to the registration of Pristine or from organic orchards in which Pristine had not been used.

To isolate *B. cinerea* from floral parts of the fruit, sepals, stamens (consisting of filaments and anthers), and styles (including stigmata) were excised from the fruit, surface disinfested in 70% ethanol for 30 s, and air dried on a sterilized paper in a laminar-flow hood. Tissues were plated onto acidified potato dextrose agar (APDA; Difco Laboratories, Detroit) (4 ml of a 25% lactic acid per liter of medium) in petri dishes. For isolation from decayed fruit, small fragments of the fruit flesh were removed from the margin of decayed and healthy tissue with a sterile scalpel and plated on APDA in petri dishes. Plates were incubated at 20°C in the dark and examined for culture development. Fungal colonies similar to those of *B. cinerea* on isolation plates were transferred onto fresh APDA. Pure cultures were then grown on PDA and incubated at 20°C under 12 h of light and 12 h of darkness to facilitate sporulation of *B. cinerea*. *B. cinerea* isolates were then single-spore cultured and mycelium plugs were stored in 15% glycerol at –80°C.

Fungicides. Technical-grade pyraclostrobin (98% active ingredient [a.i.]; BASF Corporation, Research Triangle Park, NC) and boscalid (99% a.i.; BASF Corporation) were used for in vitro assays. Pyraclostrobin and boscalid were dissolved in 100% acetone to provide stock solutions containing 10 and 25 mg/ml, respectively. Pristine is a formulated mixture consisting of one part pyraclostrobin (12.8% a.i.) and two parts boscalid (25.2% a.i.). To make a Pristine stock solution at a desired concen-

tration, one part of pyraclostrobin solution and two parts of boscalid solution at the same concentration as the desired concentration for Pristine were mixed by volume. A stock solution of salicylhydroxamic acid (SHAM, 99% a.i.; Sigma-Aldrich Inc., St. Louis) was prepared at a concentration of 100 mg/ml in methanol. For in vivo disease control studies, Pristine (38% a.i.; BASF Corporation) was used.

Sensitivity of mycelial growth. Stock solutions of technical-grade fungicides were diluted in acetone and added into PDA to produce the following concentrations: 0, 0.0005, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 µg/ml for both pyraclostrobin and Pristine and 0, 0.01, 0.05, 0.1, 0.5, 5, and 25 µg/ml for boscalid. For pyraclostrobin and Pristine, four additional concentrations of 5, 10, 50 and 100 µg/ml were prepared for testing resistant isolates or isolates with reduced sensitivity. To determine minimum inhibitory concentrations (MICs; the concentration at which fungal growth is completely inhibited) of pyraclostrobin and Pristine, isolates that were able to grow with these two fungicides at 1 µg/ml were also tested at 2, 3, 4, and 5 µg/ml. The stock solution of SHAM (1 ml) was added into 1 liter each of pyraclostrobin- and Pristine-amended media in order to inhibit the alternative oxidase respiration (26). The same amount of solvent was added into the control plates. The concentration of acetone and methanol in all media were maintained at 1 ml/liter. Single-conidial isolates were grown on PDA for 2 to 3 days at 20°C in the dark. A 5-mm-diameter plug of mycelium was removed from the margin of actively growing colonies with a sterile cork borer, placed inversely at the center of each plate, and incubated at 20°C in the dark for 3 days. Two replicate plates of each fungicide concentration were used for each isolate. The experiment was conducted twice for all isolates and three times for less-sensitive or resistant isolates. Colony diameters were measured at two perpendicular directions. EC₅₀ was calculated for each isolate.

Sensitivity of conidial germination. In all, 30 isolates (10 from each of high-, medium-, and low-EC₅₀-value groups based on the mycelial growth assay for sensitivity to boscalid) were selected to test sensitivity of conidial germination to boscalid. Ten isolates each of highly sensitive or moderately sensitive to pyraclostrobin or Pristine and four pyraclostrobin-resistant and three Pristine-resistant isolates based on the mycelial growth assay also were selected to test sensitivity of conidial germination to pyraclostrobin or Pristine. These isolates were grown on PDA in petri dishes for 3 to 4 days in the dark and then placed under 12 h of darkness and 12 h of fluorescent light (5 W m⁻²) for 2 weeks at 20°C to facilitate sporulation. To make conidial suspensions, each

plate was flooded with 15 ml of sterile deionized water, and the conidial suspensions were filtered through two layers of cheesecloth and adjusted to 2×10^5 conidia/ml with a hemacytometer. Stock solutions of fungicides were prepared as described above and added into 2% water agar (WA) to produce the following concentrations: 0, 0.005, 0.01, 0.025, 0.05, 0.075, 0.1, and 0.5 $\mu\text{g/ml}$ for pyraclostrobin; 0, 0.01, 0.05, 0.1, 0.25, 0.5, 1, and 5 $\mu\text{g/ml}$ for Pristine; and 0, 0.01, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 $\mu\text{g/ml}$ for boscalid. Additional concentrations (1, 5, 10, 50, and 100 $\mu\text{g/ml}$) for pyraclostrobin and Pristine were prepared to test less-sensitive and resistant isolates. SHAM was added into pyraclostrobin- and Pristine-amended media. The same amount of solvent was added in the control plates. A conidial suspension (100 μl) of each isolate was spread on a petri plate containing 2% WA amended with each fungicide. Two plates per replicate were used for each isolate. Plates were incubated at 20°C for 12 h. Percent germination was then determined by counting 200 conidia per plate under a microscope, and EC_{50} values were calculated. A conidium was considered germinated if the germ tube was at least the length of the conidium. The experiment was conducted twice.

Monitoring of fungicide resistance in stored apple fruit originating from orchards treated with Pristine. In November 2008, a fruit packinghouse in north-central Washington reported to us that unexpected levels of losses due to postharvest fruit rots were observed in ‘Gala’ apple fruit harvested from orchards where Pristine was applied to the fruit within 2 weeks before harvest in August 2008. The packinghouse sent us nine decayed apple fruit for diagnosis and all were caused by *B. cinerea*. To monitor whether *B. cinerea* isolates had developed resistance to pyraclostrobin, boscalid, or Pristine after use of Pristine, decayed Gala apple fruit were sampled in February 2009 from several

grower lots from the same fruit packinghouse. The orchards in which the fruit were grown in the previous year had been treated with Pristine applied within 2 weeks before harvest in each of the last 4 years. In total, 56 isolates of *B. cinerea* were obtained from decayed fruit from these grower lots. Sensitivity of mycelial growth to pyraclostrobin and Pristine and sensitivity of conidial germination to boscalid were tested as described above on the media amended with the fungicides at the MIC level (5 $\mu\text{g/ml}$). After screening the isolates at the MIC to determine their fungicide-resistance phenotypes, EC_{50} values for resistant isolates were further determined as described above.

In the baseline population study, we observed the presence of field isolates of *B. cinerea* resistant to pyraclostrobin and boscalid among the isolates sampled from an organic orchard where Pristine was not used; therefore, for comparison, in February 2009, we selected 43 isolates obtained from decayed ‘Braeburn’ apple fruit grown in the organic orchard from which resistant isolates were observed in the baseline study to determine whether the frequency of resistant isolates in Pristine-treated orchards was higher than that in the organic orchard.

Efficacy of Pristine for control of gray mold caused by fungicide-resistant and -sensitive isolates. Field experiments were conducted to determine whether resistance of *B. cinerea* to pyraclostrobin and boscalid compromised the efficacy of Pristine applied before harvest for controlling gray mold in stored apple fruit. One isolate (CLX6632) resistant to both pyraclostrobin and boscalid, one isolate (CLX6628) resistant only to pyraclostrobin, and one isolate (CLX0377) sensitive to both pyraclostrobin and boscalid were used in this study.

Experiments were conducted in two research orchards of Fuji apple trees in 2008: one with trees planted in 1993 at the Washington State University Tree Fruit Research and Extension Center (TFREC),

Wenatchee, WA and another with trees planted in 1991 at Smith Track site near Orondo, WA. In both locations, trees were under-tree irrigated. Insects and weeds were controlled following the recommendations commonly used for commercial apple production in the region (31). No fungicides were used in these two orchards after May. Pristine (0.36 g/liter of water) was applied to run-off with a powered handgun sprayer on 23 and 28 October 2008 at the TFREC and Smith Track, respectively. Control trees received no treatment. Four two-tree replicates for each treatment were arranged in a randomized complete block design. The fruit were harvested at commercial maturity 24 h after fungicide application. After harvest, fruit were immediately transported into the laboratory. There were 20 fruit per replicate. Fruit were wounded, inoculated with *B. cinerea*, and stored as described previously (34). Lesion diameters were measured at two perpendicular directions and percent fruit with gray mold within each replicate of each treatment was calculated.

Data analysis. An *F* test was used to determine whether variance of the two runs of each experiment was homogeneous and whether data could be pooled. The homogeneity of variance test indicated that the data from both runs of each experiment could be pooled ($P > 0.05$) and, thus, all further analyses were conducted on pooled data using SAS (version 9.1; SAS Institute, Inc., Cary, NC). For each isolate, a linear regression of the percent inhibition of mycelial growth or conidial germination relative to the control against the logarithm of each concentration of the fungicides was obtained using PROC REG of SAS. EC_{50} values were calculated based on the regression models. Correlation analysis was performed using PROC CORR of SAS ($P = 0.05$) to compare the sensitivity to pyraclostrobin and boscalid between mycelial growth and conidial germination and to determine whether there was cross-

Table 1. Effective concentration that inhibits fungal growth by 50% relative to the control (EC_{50}) values and minimum inhibitory concentrations (MICs) of pyraclostrobin, boscalid, and Pristine for 120 isolates of *Botrytis cinerea* from apple collected prior to the registration of Pristine or from an organic orchard where the fungicides were not used^y

Fungicide	Assay	Phenotype	No. of isolates	EC_{50} ($\mu\text{g/ml}$)		MIC ($\mu\text{g/ml}$) ^z
				Mean	Range	
Pyraclostrobin	Mycelial growth	Sensitive	116	0.043	0.008–0.132	5.0
		Resistant	4	105.250	0.520–180.3	>100
	Conidial germination	Sensitive	20	0.043	0.017–0.093	0.5
		Resistant	4	ND	0.110–>100	>100
Boscalid	Mycelial growth	–	120	5.208	0.010–69.91	>25
		Sensitive	29	0.631	0.065–1.538	5.0
	Conidial germination	Resistant	1	8.820	8.820	>25
		Sensitive	117	0.075	0.003–0.183	5.0
Pristine	Mycelial growth	Resistant	3	10.153	1.210–27.95	>100
		Sensitive	20	0.130	0.045–0.393	5.0
	Conidial germination	Resistant	3	11.090	0.650–30.85	>100
		Sensitive	3	11.090	0.650–30.85	>100

^y ND = EC_{50} ($\mu\text{g/ml}$) values for the resistant isolates were not determined because >90% of the conidia were able to germinate at the highest concentration of the fungicide that can be made in acetone; – = phenotypes were not determined based on the mycelial growth assay because the fungus was able to grow at the highest concentration used (25 $\mu\text{g/ml}$) and no definitive MIC could be established for phenotyping isolates for resistance to boscalid.

^z MIC = concentration at which the mycelial growth or germination is completely inhibited.

resistance between pyraclostrobin and boscalid.

The interaction between pyraclostrobin and boscalid in the mixture of the two active ingredients was calculated based on the EC_{50} values of conidial germination according to the following formula: $EC_{50exp} = (a + b)/[(a/EC_{50A}) + (b/EC_{50B})]$ originally proposed by Wadley (11), in which EC_{50exp} is the expected EC_{50} value from additive effects of the two components in the mixture, a and b are the absolute amounts of the components in the mixture; and EC_{50A} and EC_{50B} are the observed EC_{50} values by each of the components (A and B) independently. Synergistic interactions are present if the interaction ratio of the expected and the observed EC_{50} values is greater than 1.5; if the ratio is less than 0.5, it indicates antagonistic effects.

Data on disease control were subjected to analysis of variance using PROC ANOVA of SAS to determine whether the incidence and lesion size of gray mold differed significantly between Pristine-treated fruit and the control fruit. Means were separated using Fisher's protected least significant difference ($P = 0.05$). Data in percentage were arcsine-transformed prior to analysis (12).

RESULTS

Sensitivity to pyraclostrobin. Of the 120 isolates tested, 116 were sensitive to pyraclostrobin, with EC_{50} values ranging from 0.008 to 0.132 $\mu\text{g/ml}$ (mean = 0.043 $\mu\text{g/ml}$), and 4 isolates, which were obtained from decayed fruit from an organic orchard, were resistant to pyraclostrobin (Table 1; Fig. 1A). Of the four pyraclostrobin-resistant isolates, three were highly resistant to pyraclostrobin, with EC_{50} values of 92.5, 147.7, and 180.3 $\mu\text{g/ml}$, respectively, and resistance factors (RFs, a ratio of EC_{50} for a resistant isolate relative to the mean EC_{50} of the sensitive isolates) for these three isolates were 2,151, 3,435, and 4,193, respectively. One isolate exhibited a low level of resistance to pyraclostrobin, with an EC_{50} value of 0.52 $\mu\text{g/ml}$ (RF = 12). The MIC of pyraclostrobin for mycelial growth of sensitive isolates was 5 $\mu\text{g/ml}$, whereas resistant isolates were able to grow in the medium amended with pyraclostrobin at 100 $\mu\text{g/ml}$.

Twenty isolates sensitive to pyraclostrobin in the mycelial growth assay also were tested for sensitivity of conidial germination. The EC_{50} values of pyraclostrobin for these isolates ranged from 0.017 to 0.093 $\mu\text{g/ml}$, with a mean of 0.043 $\mu\text{g/ml}$ (Table 1). Pearson correlation analysis indicated that the EC_{50} values in the conidial germination assay were highly correlated with those in the mycelial growth assay ($r = 0.970$, $P = 0.009$).

The four isolates resistant to pyraclostrobin in the mycelial growth assay also were classified resistant to pyraclostrobin

in the conidial germination assay, with RFs ranging from 2.6 to 2,326. The MIC of pyraclostrobin for conidial germination of sensitive isolates was 0.5 $\mu\text{g/ml}$, whereas conidia of the four resistant isolates were able to germinate with pyraclostrobin at 100 $\mu\text{g/ml}$ (Table 1).

Sensitivity to boscalid. In the mycelial growth assay, the EC_{50} values of *B. cinerea* isolates sensitive to boscalid were distributed as a unimodal curve, ranging from 0.01 to 69.91 $\mu\text{g/ml}$, representing a variation factor (EC_{50} value of the least sensitive/ EC_{50} of the most sensitive isolate) of 6,991 (Table 1; Fig. 2A). No definitive MIC could be determined based on the mycelial growth assay because mycelial growth was not completely inhibited at the highest concentration used in this study (25 $\mu\text{g/ml}$).

In all, 30 isolates (10 isolates each with low, medium, and high EC_{50} values in the range of EC_{50} based on the mycelial growth assay) also were subjected to the conidial germination assay for determining EC_{50} values. Twenty-nine isolates were classified sensitive to boscalid, with EC_{50}

values ranging from 0.065 to 1.538 and a mean of 0.631 $\mu\text{g/ml}$ (Fig. 2B; Table 1). The variation factor was 24. Of the 10 isolates with high EC_{50} values in the mycelial growth assay, only 1 isolate was classified resistant to boscalid, with the EC_{50} value of 8.82 $\mu\text{g/ml}$ (RF = 14) for conidial germination. The MIC for 29 boscalid-sensitive isolates in the conidial germination assay was 5 $\mu\text{g/ml}$, whereas conidia of the isolate resistant to boscalid were able to germinate at 25 $\mu\text{g/ml}$ (the highest concentration used in this study) (Table 1). The remaining 90 isolates were sensitive to boscalid because conidia of these isolates were not able to germinate on the medium amended with boscalid at the MIC level (5 $\mu\text{g/ml}$) (data not shown).

Pearson correlation analysis of log-transformed EC_{50} values of boscalid for the 30 isolates indicated that there was no correlation ($r = 0.093$, $P = 0.626$) between EC_{50} values in the mycelial growth assay and those in the conidial germination assay.

Sensitivity to Pristine. Of the 120 isolates tested, 117 were sensitive to Pristine,

with EC₅₀ values ranging from 0.003 to 0.183 µg/ml (mean = 0.075 µg/ml) (Table 1; Fig. 1B). Two isolates resistant to pyraclostrobin but sensitive to boscalid were moderately resistant to Pristine in the mycelial growth assay, with EC₅₀ values of 1.21 (RF = 16) and 1.30 µg/ml (RF = 17), respectively. One isolate resistant to both pyraclostrobin and boscalid was highly resistant to Pristine, with an EC₅₀ value of 27.95 µg/ml (RF = 373).

The EC₅₀ values of Pristine for 20 isolates sensitive to Pristine in the conidial germination assay ranged from 0.045 to

0.393 µg/ml, with a mean of 0.13 µg/ml, which was higher than that in the mycelial growth assay (Table 1). The Pristine-resistant isolates classified based on the mycelial growth assay also were classified resistant to Pristine based on the conidial germination assay. The MICs of Pristine for both mycelial growth and conidial germination of sensitive isolates were 5 µg/ml, whereas resistant isolates were able to grow or germinate with Pristine at 100 µg/ml (Table 1).

The tests with the mixture of technical-grade pyraclostrobin and boscalid at 1:2

ratio for conidial germination of *B. cinerea* indicated that there was an additive interaction between the two fungicides, with the interaction ratios between 0.8 and 1.2 in all five isolates tested (Table 2). No synergistic or antagonistic interactions were observed.

Cross-sensitivity between pyraclostrobin and boscalid. The log-transformed EC₅₀ values of boscalid based on the conidial germination assay for the 36 isolates (30 from the baseline population and 6 resistant to either pyraclostrobin or boscalid from the monitoring study) were plotted versus the log-transformed EC₅₀ values of pyraclostrobin based on the mycelial growth assay and analyzed using the CORR procedure of SAS. Pearson correlation analysis indicated that there was no significant correlation in sensitivity ($r = -0.044$, $P = 0.80$) between pyraclostrobin and boscalid (Fig. 3).

Monitoring of resistance in *B. cinerea* populations on stored apple fruit originating from orchards treated with Pristine. Of the 56 isolates obtained from decayed apple fruit originating from conventional orchards in which Pristine had been used for four consecutive years, 11 isolates (19.6%) were resistant to both pyraclostrobin and boscalid and 1 isolate was resistant only to pyraclostrobin (Tables 3 and 4). Of the 43 isolates collected in February 2009 from decayed apple fruit that originated from the organic orchard where resistant isolates were detected in the baseline study, 2 (4.7%) were resistant to both pyraclostrobin and boscalid, 3 were resistant only to pyraclostrobin, and 2 were resistant only to boscalid. The isolates resistant to both pyraclostrobin and boscalid also were resistant to Pristine, with the EC₅₀ values ≥ 30.6 µg/ml. The isolates resistant only to boscalid were sensitive to Pristine. However, the isolates resistant only to pyraclostrobin exhibited a low or moderate level of resistance to Pristine, with the EC₅₀ values ranging from 0.44 to 2.49 µg/ml (Table 4).

Efficacy of Pristine for control of gray mold caused by fungicide-resistant and -sensitive isolates. The results from the experiments conducted in the two orchards were consistent. Pristine applied to the fruit 1 day before harvest reduced both the lesion size and incidence of gray mold on the fruit inoculated with the isolate (CLX0377) sensitive to both pyraclostrobin and boscalid (Table 5). The magnitude of reduction in gray mold incidence and lesion size between the nontreated and Pristine-treated fruit was similar to that previously reported on Fuji apple fruit (34). In contrast, Pristine failed to control gray mold on the fruit inoculated with the isolate (CLX6632) resistant to both pyraclostrobin and boscalid (Table 5). On the fruit inoculated with the isolate CLX6628, which was resistant to pyraclostrobin but sensitive to boscalid, Pristine reduced gray

Fig. 2. Frequency distributions of effective concentration that inhibits fungal growth by 50% relative to the control (EC₅₀) values of boscalid based on **A**, a mycelial growth assay for 120 isolates and **B**, a conidial germination assay for 30 isolates of *Botrytis cinerea* from apple collected prior to the registration of Pristine or from an organic orchard where the fungicides were not used.

Table 2. Interaction between pyraclostrobin and boscalid in the mixture of the two fungicides at 1:2 ratio in conidial germination experiments for *Botrytis cinerea* from apple

Isolate	Observed EC ₅₀ value (µg/ml)			EC _{50exp} ^y	Interaction ^z
	Pyraclostrobin	Boscalid	Pyraclostrobin + boscalid		
CLX1764	0.034	0.922	0.118	0.095	0.8
CLX1922	0.017	1.476	0.045	0.050	1.1
CLX1927	0.052	0.919	0.121	0.140	1.2
CLX6698	0.069	0.378	0.131	0.152	1.2
CLX6703	0.020	0.122	0.047	0.045	1.0

^y EC_{50exp} is the expected effective concentration at which a mixture of pyraclostrobin and boscalid at 1:2 ratio inhibits conidial germination by 50% relative to the control.

^z Ratio of expected (EC_{50exp}) and observed EC₅₀ (pyraclostrobin + boscalid) values.

mold lesion size at both experimental sites, and gray mold incidence by approximately 18% relative to the nontreated control at the TFREC site but not at the Smith Track site.

DISCUSSION

In this study, we established the distribution of baseline sensitivity to boscalid, pyraclostrobin, and Pristine based on field isolates of *B. cinerea* from apple collected prior to the registration of the fungicides for commercial use or from orchards where the fungicides were not used. We found that field populations of *B. cinerea* from apple in the region consisted of phenotypes varying in degrees of sensitivity to these fungicides. Monitoring of fungicide resistance indicated that approximately 20% of the isolates recovered from decayed apple fruit from orchards where Pristine was used for four consecutive years exhibited resistance to both boscalid and pyraclostrobin as well as resistance to Pristine. This is the first report of occurrence of dual resistance to boscalid and pyraclostrobin in field isolates of *B. cinerea*.

Previous studies have demonstrated that spore germination is the stage of fungal development that is particularly sensitive to pyraclostrobin and other strobilurin fungicides, though strobilurin fungicides also are effective against fungal mycelial growth (3). Conidial germination has been used in assessing sensitivity of fungal pathogens to strobilurin fungicides (2,24,25). In our study, sensitivity of mycelial growth of *B. cinerea* to pyraclostrobin appeared to be consistent with that of conidial germination. Four isolates resistant to pyraclostrobin based on the mycelial growth assay showed a similar level of resistance in the conidial germination assay. There was no difference in the mean EC_{50} values of the sensitive isolates between mycelial growth and conidial germination assay. With the mycelial growth assay, we were able to determine a definitive MIC of pyraclostrobin against mycelial growth of *B. cinerea* because all sensitive isolates were unable to grow on the medium amended with pyraclostrobin at 5 $\mu\text{g/ml}$. Because mycelial growth assay is relatively easier and less time-consuming than the conidial germination assay, we recommend that the mycelial growth assay with pyraclostrobin at 5 $\mu\text{g/ml}$ be used for phenotyping *B. cinerea* isolates for resistance to pyraclostrobin.

In our study, all four isolates resistant to pyraclostrobin within the baseline populations were obtained from an organic orchard that had been certified for organic production since the mid-1990s. It is unlikely that strobilurin fungicides had been used in this orchard prior to transition to organic production because the first strobilurin fungicide (kresoxim-methyl) for use on apple was registered in the mid-1990s. However, this orchard is sur-

rounded by other apple orchards. Strobilurin fungicides such as kresoxim-methyl and trifloxystrobin may have been used in these surrounding orchards before they were certified in the early 2000s for organic production. It may be possible that QoI-resistant isolates of *B. cinerea* existed in these orchards and could have spread into adjacent orchards because spores of *B. cinerea* can be dispersed by wind.

Baseline sensitivity of *B. cinerea* to boscalid has also been reported in three other countries (25,33,35). It appeared that EC_{50} values of boscalid against mycelial growth of *B. cinerea* varied considerably among these studies, including the present study. The lowest EC_{50} values were similar in all studies, including ours, but the highest values varied among the studies, ranging from 0.21 to 69.91 $\mu\text{g/ml}$. These differences in EC_{50} values may be due to the differences in the growth substrates used in different studies. Stammler and Speakman (33) used a liquid YBA medium and Myresiotis et al. (25) used a minimal medium in the tests on sensitivity of mycelial growth of *B. cinerea* to boscalid whereas, in the present study and that of Zhang et al. (35), the tests were done using PDA. It seemed that *B. cinerea* was more sensitive to boscalid on YBA than on the minimal medium or PDA. Leroux (19) reported that the activity of boscalid significantly increased in the presence of succinate or acetate instead of glucose. In our study, great variability in EC_{50} values of boscalid on mycelial growth was observed. In addition, no definitive MIC can be determined based on the mycelial growth assay when the highest concentration of boscalid (25

$\mu\text{g/ml}$) was used in this study because mycelial growth of *B. cinerea* was not completely inhibited at this concentration. Furthermore, of the 10 isolates with EC_{50} values >10 $\mu\text{g/ml}$ in the mycelial growth assay, only 1 isolate was confirmed as resistant to boscalid in the conidial germination assay. Similarly, Myresiotis et al. (25) reported that mycelial growth of *B. cinerea* was not completely inhibited by boscalid at the highest concentration they tested (5 $\mu\text{g/ml}$). Taking these findings together, it appeared that the mycelial growth assay is not suitable for phenotyping *B. cinerea* isolates for resistance to boscalid.

In the present study, conidial germination of *B. cinerea* was more sensitive to boscalid than mycelial growth. This is consistent with the results of Zhang et al. (35), who reported that the mean EC_{50} values of boscalid for mycelial growth of *B. cinerea* was higher than that for conidial germination. However, Myresiotis et al. (25) reported that there was no difference in the range and mean EC_{50} values of boscalid against *B. cinerea* between mycelial growth and conidial germination assays. This is likely due to different media used in these studies because media does affect mycelial growth assays, as discussed above. In our study, the conidial germination assay yielded consistent results. In the baseline population, conidial germination of all sensitive isolates was completely inhibited on PDA amended with boscalid at 5 $\mu\text{g/ml}$, whereas the resistant isolate CLX6632 germinated well at this concentration and even with boscalid at 25 $\mu\text{g/ml}$. Furthermore, Pristine at label rate failed to

Fig. 3. Correlation between sensitivity to pyraclostrobin based on the mycelial growth assay and sensitivity to boscalid based on the conidial germination assay using 36 *Botrytis cinerea* isolates (30 isolates from the baseline population and 6 isolates resistant to either pyraclostrobin or boscalid from the monitoring population) from apple. EC_{50} = effective concentration of a fungicide that inhibits fungal growth by 50% relative to the control.

Table 3. Monitoring of resistance to pyraclostrobin and boscalid in field isolates of *Botrytis cinerea* obtained from decayed apple fruit

Orchard type, grower lot (orchard) ²	No. of isolates	No. of isolates resistant to ^y		
		Pyraclostrobin only	Boscalid only	Pyraclostrobin and boscalid
Conventional				
C1	9	0	0	2
C2	2	1	0	1
C3	12	0	0	1
C4	24	0	0	4
C5	1	0	0	1
C6	8	0	0	2
Total	56	1	0	11
Organic				
O1	43	3	2	2

^y Resistance phenotypes were determined at a discriminatory concentration of 5 µg/ml on agar media in a conidial germination assay for boscalid and a mycelial growth assay for pyraclostrobin and Pristine. Sensitive isolates were completely inhibited at the discriminatory concentration, whereas resistant isolates were able to grow or germinate.

² In conventional apple orchards, Pristine had been used for four consecutive seasons since 2005, whereas no synthetic fungicides were used in the organic orchard since the mid-1990s. Fruit were grown in the orchards during the 2008 growing season, and decayed fruit were collected during storage in November 2008 and February 2009 from fruit packinghouses.

Table 4. Effective concentration that inhibits fungal growth by 50% relative to the control (EC₅₀) values and phenotypes of resistance to pyraclostrobin, boscalid, and Pristine in field isolates of *Botrytis cinerea* collected from decayed apple fruit

Orchard type, isolate ²	EC ₅₀ (µg/ml) and phenotype ^y		
	Pyraclostrobin	Boscalid	Pristine
Conventional			
CLX7391	545.75 (R)	42.49 (R)	252.98 (R)
CLX7392	804.26 (R)	17.52 (R)	52.84 (R)
CLX7451	305.93 (R)	12.12 (R)	163.00 (R)
CLX7452	41.55 (R)	0.11 (S)	0.44 (R)
CLX7453	9,582.31 (R)	248.79 (R)	194.81 (R)
CLX7455	480.52 (R)	9.36 (R)	243.67 (R)
CLX7465	933.39 (R)	48.38 (R)	177.16 (R)
CLX7466	3,067.66 (R)	121.69 (R)	485.41 (R)
CLX7474	188.00 (R)	6.39 (R)	69.38 (R)
CLX7477	630.84 (R)	6.42 (R)	114.45 (R)
CLX7491	1,041.72 (R)	186.67 (R)	88.53 (R)
CLX7494	79.63 (R)	7.20 (R)	30.60 (R)
Organic			
CLX7420	2,850.27 (R)	51.00 (R)	98.60 (R)
CLX7425	237.25 (R)	0.28 (S)	1.20 (R)
CLX7426	70.09 (R)	0.23 (S)	2.21 (R)
CLX7434	0.01 (S)	116.67 (R)	0.04 (S)
CLX7435	493.86 (R)	0.65 (S)	2.49 (R)
CLX7436	0.04 (S)	61.82 (R)	0.10 (S)
CLX7437	592.30 (R)	17.44 (R)	127.59 (R)

^y EC₅₀ values of pyraclostrobin and Pristine were estimated in a mycelial growth assay. EC₅₀ values of boscalid were estimated in a conidial germination assay. Resistance phenotypes were determined at a discriminatory concentration of 5 µg/ml on agar media based on a conidial germination assay for boscalid and a mycelial growth assay for pyraclostrobin and Pristine. Sensitive isolates were completely inhibited at the discriminatory concentration, whereas resistant isolates were able to grow or germinate. Phenotypes are listed in the parentheses: R = resistant and S = sensitive.

² In conventional apple orchards, Pristine had been used for four consecutive seasons since 2005, whereas no synthetic fungicides were used in the organic orchard since the mid-1990s. Fruit were grown in the orchards during the 2008 growing season, and decayed fruit were collected during storage in November 2008 or in February 2009 from fruit packinghouses.

control gray mold on the fruit inoculated with CLX6632. Therefore, we recommend that boscalid at 5 µg/ml be used as a discriminatory concentration in the conidial germination assay for phenotyping *B. cinerea* isolates for resistance to boscalid. Using this approach, we were able to separate boscalid-resistant isolates from sensitive isolates in the monitoring of fungicide resistance in the field as documented in this study.

Previous reports based on laboratory-induced fungicide-resistant mutants of *B. cinerea* indicated that pyraclostrobin-resistant mutants were more sensitive to boscalid than the wild-type isolates (21) and that boscalid-resistant mutants showed an increased sensitivity to the QoI fungicides kresoxim-methyl and azoxystrobin (35). However, in our study, we found that there was no negative cross-resistance between the QoI pyraclostrobin and the

SDHI (carboxamide) boscalid in field resistant isolates of *B. cinerea*. Furthermore, in our study, although phenotypes with resistance to both fungicides occurred in the populations of *B. cinerea* from stored apple fruit, phenotypes that were resistant only to either pyraclostrobin or boscalid also occurred in the populations. These results suggest that there was no cross-resistance between pyraclostrobin and boscalid in *B. cinerea*. Our findings are consistent with those of Avenot et al. (2), who reported that no cross-resistance between pyraclostrobin and boscalid was observed in field isolates of *A. alternata* from pistachio. The discrepancy between our results and those from the previous reports based on laboratory mutants may be due to the differences in the selection process between laboratory mutants and field resistant isolates. Fungicide resistance phenotypes of laboratory-induced mutants may or may not become established under commercial field conditions, depending on the presence of selection pressure and stability, fitness, and competitive ability of the mutants.

A high frequency (approximately 20%) of dual resistant isolates was observed among the *B. cinerea* isolates obtained from gray-mold-decayed apple fruit from commercial apple orchards where Pristine had been used, whereas all isolates collected during 2001–03, prior to the registration of Pristine in 2005, were sensitive to both pyraclostrobin and boscalid, suggesting that the use of Pristine in four consecutive years in these orchards had imposed selection pressure for development of resistance to the two fungicides. Because no cross resistance between pyraclostrobin and boscalid was observed among the solo-resistant isolates tested in this study, it appears that the phenotype with resistance to both pyraclostrobin and boscalid is apparently a result of multidrug resistance. It was unexpected to see such a quick development of resistance in *B. cinerea* to both pyraclostrobin and boscalid, given the fact that only one application of Pristine was made in each crop season in these orchards. Pristine was applied within 2 weeks before harvest in these orchards for controlling postharvest gray mold and blue mold on the fruit. However, other QoI fungicides were commonly used in these orchards for controlling powdery mildew in the early growing season. The use of QoI fungicides for control of other diseases in the orchard could increase the selection pressure for the development of resistance to QoIs in *B. cinerea* populations. The presence of QoI-resistant strains in a fungal population could increase the likelihood of development of resistance to boscalid when the boscalid-containing product Pristine is used. Such a case has been observed on *A. alternata* on pistachio (2). Furthermore, some of these orchards also are next to sweet cherry orchards

Table 5. Incidence and lesion size of gray mold on Fuji apple fruit treated with Pristine (pyraclostrobin + boscalid) 1 day before harvest in two orchards, inoculated at harvest with *Botrytis cinerea* isolates resistant or sensitive to the two fungicides, and stored at 0°C for 8 weeks^x

Isolate	Phenotype ^z	Treatment	Orchard at TFREC ^y		Orchard at Smith Track	
			Incidence (%)	Lesion size (mm)	Incidence (%)	Lesion size (mm)
CLX0377	Pyr ^S Bos ^S	Control	97.5 a	52.9 a	96.3 a	47.0 a
	...	Pristine	22.5 b	28.6 b	36.3 b	23.6 b
CLX6628	Pyr ^R Bos ^S	Control	100.0 a	85.6 a	98.8 a	84.4 a
	...	Pristine	82.5 b	31.3 b	97.5 a	32.5 b
CLX6632	Pyr ^R Bos ^R	Control	100.0 a	77.8 a	100.0 a	60.6 a
	...	Pristine	100.0 a	80.5 a	100.0 a	66.8 a

^x Average lesion size within each replicate of each treatment was calculated among the decayed fruit only. Values followed by the same letter within a column within the same isolate are not significantly different according to Fisher's protected least significant difference at $P = 0.05$.

^y Washington State University Tree Fruit Research and Extension Center.

^z Pyr = pyraclostrobin, Bos = boscalid, S = sensitive, R = resistant.

where QoI fungicides, including pyraclostrobin and Pristine, were commonly used to control cherry powdery mildew. Because *B. cinerea* also is commonly present in sweet cherry orchards, fungicides used for cherry mildew control could create selection pressure for development of resistance to QoIs and boscalid in *B. cinerea*. *B. cinerea* from sweet cherry orchards could be spread into apple orchards next to the cherry orchards. No information is available about the impact of fungicide usage patterns on the development of resistance to QoIs and boscalid in *B. cinerea* in sweet cherry orchards and their potential role in providing fungicide-resistant inoculum to apple orchards next to the cherry orchards.

Pristine applied at the label rate failed to control gray mold caused by the isolate resistant to both pyraclostrobin and boscalid, suggesting that the build-up of dual-resistance phenotypes could result in the failure of disease control in commercial operations because the phenotype with resistance to both fungicides was common among the isolates obtained from decayed apple fruit from orchards where Pristine was used. In the in vivo study, Pristine also failed to reduce incidence of gray mold on the fruit incited by the isolate resistant to pyraclostrobin but sensitive to boscalid. This is consistent with the results from in vitro sensitivity tests, which showed that the isolates resistant to pyraclostrobin but sensitive to boscalid exhibited a low level of resistance to Pristine, whereas the isolates resistant to boscalid but sensitive to pyraclostrobin were sensitive to Pristine. Similarly, Avenot et al. (2) also reported that Pristine and Endura (boscalid) failed to control the disease caused by Pristine-resistant isolates of *A. alternata* in pistachio. The failure of Pristine to control gray mold caused by the isolate resistant only to pyraclostrobin could be due to a low level of boscalid residue present on or in the fruit. The MIC against conidial germination of *B. cinerea* was 5 and 0.5 µg/ml for boscalid and pyraclostrobin, respectively. The boscalid residue on or in the fruit at harvest might be lower than the MIC, resulting in unsuccessful inhibition of conidia germination and, thus, leading to

decay development. However, we did not monitor fungicide residues on or in the fruit in the current study.

In summary, in this study, we assessed baseline sensitivity to pyraclostrobin and boscalid in *B. cinerea* populations from stored apple fruit and established methods for phenotyping *B. cinerea* isolates for resistance to these two fungicides. Monitoring of resistance indicated that a high frequency of dual resistance to pyraclostrobin and boscalid was observed among the isolates obtained from decayed fruit sampled from a fruit packinghouse. However, occurrence and frequency of resistant isolates in apple orchards still remains to be determined. Development of a fungicide-resistant population that may result in the failure of disease control is dependent on the presence of selection pressure and stability, fitness, and competitive ability of resistant phenotypes. Future research is needed to determine whether phenotypes reported in this study are stable and whether fitness costs are associated with them. Such information would help develop strategies or tactics for fungicide resistance management.

ACKNOWLEDGMENTS

This research was supported, in part, by the Washington Tree Fruit Research Commission. We thank the packinghouses involved in the collection of apple samples. This manuscript is Plant Pathology New Series 0535, Department of Plant pathology, College of Agricultural, Human, and Natural Resource Sciences Agricultural Research Center, Project No. WNP00367, Washington State University, Pullman.

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