Analysis of boscalid resistance of isolates of *Botrytis cinerea*

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Analysis of boscalid resistance of isolates of *Botrytis cinerea*

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Abstract

Succinate dehydrogenase (Sdh) inhibitor fungicides, such as boscalid, are effective for the management of gray mold caused by the fungal pathogen *Botrytis cinerea*. Unfortunately, resistance to boscalid was common among isolates of the pathogen from small fruits grown in Oregon. Boscalid-resistance is commonly associated with mutations in Sdh, especially in subunit B. *Sdh*B was sequenced from five boscalid-sensitive and five boscalid-resistant isolates of *B. cinerea.* A majority of the boscalid-resistant isolates had a single nucleotide polymorphism at codon 272 resulting in a substitution of histidine with arginine (H272R). The boscalid-sensitive isolates did not harbor this mutation. The transposons *Boty* and *Flipper* also were found within the genomes of the resistant strains, but not boscalid-sensitive isolates. Additionally, a pattern was found to exist between the transposons present within the strains and source location. Additional studies with a greater number of isolates are required to verify this correlation.

Keywords: Gray mold, fungicide resistance, SdhB, *Boty*, *Flipper*

Introduction

Botrytis cinerea is regarded as the second most important fungal plant pathogen for its scientific and economic importance (Dean *et al*. 2012). *B. cinerea* competes with the plant for nutrients, decaying the fruit and ultimately killing it. Hosts will experience skin thinning and gray mold on their surface. *B. cinerea* is capable of infecting all parts of the plant except for the

roots. Additionally, the pathogen has a broad host range, with records on over 500 plant genera (Dean *et al*., 2012; Elad *et al*. 2016), including important agronomic crops such as grapes. Thus, the economic impact of *Botrytis cinerea* is high, reaching numbers of up to $100 billion in losses worldwide (Dean *et al*. 2012).

In Oregon, small fruit production comprises a large sector of the agricultural economy. Unfortunately, the small fruits are prone to infection by *B. cinerea*. Farmers use commercial products such as fungicides to inhibit the growth of *B. cinerea.* However, fungicides can present environmental concerns due to residues left on crops (Aubee and Lieu 2010); thus optimization of strategies to control gray mold are necessary *(*Fillinger & Walker 2016). Previous research found that 64% of isolates collected from small fruit fields were resistant to two or more fungicides (Stockwell *et al*. 2018). This study will examine resistance to boscalid in depth, including the discriminatory dose and the mechanism of resistance to this fungicide.

Boscalid is a fungicide belonging to the group of succinate dehydrogenase inhibitors that interfere with the cellular respiration of pathogenic fungi (Dehne *et al.* 2010). An international group of scientists serving on the Fungicide Resistance Action Committee (FRAC) classifies fungicides based on mode of action. Boscalid is in FRAC group 7, targeting the succinate dehydrogenase complex (Sdh) that is bound to the inner mitochondrial membrane. Sdh plays a vital role in the electron transport chain and Kreb's Cycle. The electron transport chain is a series of oxidations and reductions that occur in order to create a proton motive force that is used for the generation of adenosine triphosphate (ATP) (Aubee and Lieu, 2010). Organisms can then use ATP to drive many cellular functions. Succinate dehydrogenase is one the four membrane multi-subunit protein complexes involved in the electron transport chain. Additionally, there are 2 mobile electron transporters known as coenzyme Q and cytochrome c. In the first step of the electron transport chain, coenzyme Q receives electrons from the oxidation of NADH through complex one. Then, coenzyme Q receives electrons through Sdh from the oxidation of FADH2 (Aubee and Lieu 2010). When Sdh is inhibited, coenzyme Q is unable to shuttle electrons through the membrane, and thus the electron transport chain cannot proceed to create the ATP that the fungi need to respire and live.

Resistance can occur through six known mechanisms; target overproduction, activation of an alternative pathway compensating for the inhibition, increase expulsion of the fungicide from the system, decrease fungicide concentration within the cell, and modification of target site

proteins. Modifications to target proteins are the most common mechanisms of resistance in strains of *Botrytis cinerea* (Fillinger and Walker, 2016). As outlined before, boscalid targets Sdh and inhibits the cellular respiration of the fungi. Sdh is composed of four different subunits; SdhA, SdhB, SdhC, and SdhD *(Leroux et al. 2010)*. A mutation to any of the subunits could interfere with the binding of boscalid to Sdh. Research suggests that a correlation between mutations occurring within the *sdhB* gene and resistance classification exists (Fernandez-Ortuño *et al.* 2017; Lateve *et al*. 2014; Veloukas *et al.* 2013).

Leroux *et al.* (2010) among other sources found that a majority of the boscalid-resistant strains of *B. cinerea* collected from French and German vineyards had single nucleotide polymorphisms in the gene encoding the SdhB subunit, frequently in codon 272. An objective of this study is to determine if the same polymorphisms are found in strains of *B. cinerea* collected from small fruit fields in Oregon.

The genetic diversity of *B. cinerea* can be better understood by tracing the migration of specific genetic markers. Ma and Michailides (2005) identified two unique transposons within *B. cinerea* in California; *Boty* and *Flipper*. Detecting the transposons within the strains tested can illustrate the genetic diversity of *B. cinerea* present in the small fruit fields of Oregon. An objective of this study is to analyze isolates in Oregon for the presence of the *Boty* and *Flipper* transposons.

Materials and Methods

Isolation of *Botrytis* from berries. A representative sample of 50 berries was collected from each field that was sampled. The berries were collected evenly throughout the rows. Berries in groups of ten were wrapped in nylon mesh and soaked in 10% commercial bleach (BI-MART, Eugene, OR) for 10 minutes, then in 70% ethanol for 1 minute, followed by two rinses in sterile deionized water. The berries were then placed on a lava rock radiant support tray with holes to reduce movement of the berries within an aluminum grill tray with measurements of 18 x 3.5 x14 inches at equal distances from each other. Two autoclaved paper towels soaked with deionized water were placed at the bottom of the foil tray. The outside of the tray was wrapped with plastic wrap to maintain a moist environment for fungal growth and maintained at room temperature for about 7 days. Observations were made every 24 hours after incubation. From each of the berries with symptoms of gray mold, the outgrowing fungus was transferred to ¼

strength potato dextrose agar (Difco Laboratories, Sparks MD) amended with streptomycin (Sm) at 100 mg/ml to prevent bacterial growth. The fungus was then transferred a second time prior to storage at -80°C in cryovials containing nutrient broth amended with 20% (v/v) glycerol. Preliminary identification of isolates as *Botrytis* was done through observing hyphal morphology, sclerotia formation, and conidia production.

***Botrytis* isolates selected for boscalid resistance and molecular characterization.** Ten isolates of *Botrytis* collected from small fruit fields in Oregon in 2014 to 2017 were selected for characterization (Table I). Five of the isolates were previously scored as sensitive to boscalid and five others were scored as resistant based on their ability to grow on culture media amended with 5 ppm boscalid (personal communication, Stockwell).

Boscalid discriminatory dose assay. A commercial formulation of boscalid (Endura, 70% w/w, BASF Corporation, Florham Park, New Jersey) was used to determine sensitivity to the active ingredient of the fungicide. A stock solution containing 10,000 ppm boscalid was made. Different quantities of the stock solution were added to autoclaved and molten 0.5% Yeast Extract Agar (YEA) to final concentrations of 0.00, 0.01, 0.10, 1.00, 10.00, and 100.00 mg/L of boscalid. The 0.5% YEA medium that was not amended with boscalid was the negative control. The 0.5% YEA medium amended with 100 mg/L of boscalid was the positive control.

Botrytis isolates were retrieved from -80ºC storage and placed on ¼ strength potato dextrose agar (PDA) amended with 100 mg/ml Sm. Isolates were then transferred onto cyprodinil test agar (CTA) plates. After sufficient growth, 4 mm diameter plugs with actively growing hyphae were cut from the medium with a cork borer and transferred onto the test medium of 0.5% Yeast Extract (Difco laboratories, Sparks, MD) solidified with 1.8% Bacto-agar (Difco laboratories, Sparks, MD) and amended with different concentrations of boscalid. Three plates per concentration were used for each isolate. The plates were incubated at 23°C. The diameter growth of the isolates was measured daily from 2 to 5 days with aid of a digital caliper (EZ Cal, iGaging, San Clemente, CA). The experiment was repeated once.

The effective concentration that inhibited at least 50% growth (EC50) of each isolate was calculated using the BioQuest online calculator (https://www.aatbio.com/tools/ec50-calculator).

Data combined from two experiments of an isolate were uploaded onto the BioQuest calculator. EC50 values were provided by the website.

DNA extraction. The method of Cenis (1992) was used to extract DNA from fungal cultures, with minor modifications. Hyphae were collected from isolates grown on 0.5% YEA medium at 18° C. Briefly, hyphae of *B. cinerea* were added directly to 300 µl extraction buffer (200 mM Tris HC1 pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) in a 1.5 ml Eppendorf tube. The hyphae were disrupted with a hand-held conical grinder for 2 minutes, and 150 µl of 3 M sodium acetate, pH 5.2 was added. The samples were incubated at -20 ºC for 10 minutes, then centrifuged at 2,016 x g at room temperature for five minutes. A 400 µl sample of the supernatant was transferred to another tube and an equal volume of isopropanol was added. After incubation for 5 minutes at room temperature, the suspension was subjected to centrifugation for 15 minutes at 10,976 x g. The pellet of precipitated DNA was washed with 70% ethanol, air dried for an hour, and resuspended in 50 µl of Tris EDTA. DNA purity and concentration was measured with a nanodrop spectrophotometer (Thermo Fischer Scientific, Waltham MA).

PCR. The primers used for amplification of the transposases *Flipper* and *Boty* are listed in Table II and were designed by Ma and Michailides (2005). Taq polymerase (Thermo Fischer Scientific, Waltham MA) was used for the amplification process. Each PCR reaction contained a total volume of 25 µl and was run in a Veriti model 9902 thermal cycler (Applied Biosystems, Waltham, MA).

For the *Flipper* PCR, 0.5 µl of the 10µM F300F primer, and 0.5 µl of 10µM F1550R primer were added. The conditions for amplification of *Flipper* with Taq polymerase were initial denaturation at 95°C for 3 minutes, then 40 cycles of 95° for 30 seconds, 60°C for 40 seconds, 72°C for 75 seconds, and a final extension at 72°C for 10 minutes.

For the *Boty* PCR, 0.5 µl of 10µM BotyF4 primer and 0.5 µl of 10µM BotyR4 primer were added. The same conditions used for *Flipper* amplification were used for *Boty*, except the annealing temperature was 68°C.

For *sdhB*, 0.5 µl of the forward primer 10µM IpBcBeg and 0.5 µl of the reverse primer 10µM IpBcEnd2 were added to the PCR mixture. Amplification of *sdhB* with Taq polymerase was performed under the following conditions; initial denaturation at 95°C for 3 minutes then 35 cycles at 95° for 30 seconds, 60°C for 30 seconds, 68°C for 1 minute and a final extension at 68°C for 4 minutes.

Gel electrophoresis. Five µl of each PCR reaction was combined with 2 µl of 6X loading buffer, and 5µl of sterile milliQ water and loaded onto a lane in a 1% (w/v) agarose gel containing ethidium bromide. A 1 KB plus ladder (Thermo Fischer Scientific, Waltham MA) was added to a separate lane and used to estimate amplicon size. The gel was electrophoresed in TAE (Tris base, acetic acid and EDTA) buffer and run at 100V. DNA in the gel was visualized and photographed with a UV imager (Alpha Innotech, San Leandro, CA).

Sequencing of *sdhB* and analysis. Each of the reactions for *sdhB* yielded single amplicons. The remaining PCR reaction was treated with ExoSAP-IT and sequenced by the Oregon State University, Center for Genome Research and Biocomputing, Core Facilities, Corvallis, Oregon. Sequence analysis of *sdhB* involved aligning the forward and reverse *sdhB* sequences of the 10 isolates using the Geneious Bioinformatics Software to generate a consensus sequence each *sdhB* gene. The consensus nucleotide sequences of each isolate was aligned to the reference sequence of the *sdhB* gene for *Saccharomyces cerevisiae*. Regions of high uncertainty were not used. The consensus was then translated into a predicted amino acid sequence. The frame for translation chosen was based on the alignment of the reference sequence to those of *Botrytis*.

Results

***Botrytis* isolates differed in sensitivity to boscalid.** The 10 isolates grown on 0.5% YEA medium amended with boscalid were classified as resistant or sensitive based on their observed growth at 1 ppm. Isolates that achieved more than 50% growth were classified as resistant. Isolates that achieved 50% or less growth were classified as sensitive. Originally, different concentrations were tested to determine the EC50 of each isolate. EC50 values were to be used to classify isolates but the results of the different trials for each isolate varied significantly as illustrated in Table I. Mean values were obtained by averaging EC50 values for each isolate across two experiments. Standard deviation was calculated by squaring the differences between the calculated EC50 values for each isolate and the calculated mean for that isolate. Then, the mean of the squared differences was calculated. The square root of those values were then taken. A standard x^2 test was used to determine significance with a 95% confidence level. Observed growth at 1 and 100ppm were used to make profile classifications. Growth of the isolates relative

to the medium lacking fungicide are shown in Table III, and their resistance classifications are presented in Table IV.

***Boty* and *Flipper* transposons are detected in some *Botrytis* isolates. Isolates were classified as being of type** *vacuma* or *transposa. Vacuma* isolates did not contain any of the transposable elements, *Boty* or *Flipper*. *Transposa* isolates contained both *Boty* and *Flipper*. Sixty percent of the isolates contained at least one of the transposable elements. Fifty percent of the isolates were of the type *transposa*. Forty percent of the isolates were of the type *vacuma* as illustrated in Table IV. A correlation was found between the presence of the transposons and the county from which they were collected. *B. cinerea* isolates collected from Polk county were of the type *vacuma*. Isolates collected from Washington and Clackamas were of the type *transposa.*

***SdhB* gene sequence analysis reveals a positive correlation between amino acid present at codon 272 and resistance classification.** Consensus identities between the *sdhB* gene of the 10 isolates revealed that there was a correlation between the amino acid present at a codon 272 and boscalid resistance classification. The variance was due to either the presence of histidine or arginine at that position. Isolates with the arginine at codon 272 were classified as resistant, while the isolates with the histidine at the same position were classified as sensitive.

Discussion

The EC50 values for sensitive isolates ranged from 0.12 mg/L to 1.02 mg/L. In contrast, the EC50 values for sensitive isolates classified by Myresiotis (Myresiotis *et al.* 2008), who also measured hyphae growth, ranged from 0.075 to 5.05 mg/L. For resistant isolates, the EC50 values ranged from 0.43 mg/L to 34.84 mg/L. The range for the sensitive and resistant isolates overlapped signifying that perhaps a discriminatory dose would be a more appropriate tool to classify the isolates as sensitive or resistant. Thus, observed growth at 1and 10 ppm was the method chosen to classify the isolates as sensitive or resistant.

The results of the *sdhB* sequence analysis are consistent with those of Leroux *et al.* (2010), Veloukas *et al.* (2013) Lateve *et al*. (2014) and Fernandez-Ortuño *et al.* (2017) who determined that there was a high correlation between the type of amino acid found at codon position 272 and the sensitivity or resistance to boscalid of the isolate. DR-26-15-P6-3A was the

only isolate that did not support the correlation found. However, isolate DR-26-15-P6-3A was close to being classified as sensitive by achieving only 52% growth at 1 and 10 ppm. An arginine at codon 272 correlated with a resistant classification. The resistance could be due to a change in the physical structure of the SdhB subunit that weakens the ability of boscalid to bind to it. Therefore, if boscalid is unable to bind to the SdhB subunit it is unable to inhibit the respiration of the *B. cinerea* strain, and overall growth. Potentially, other FRAC 7 fungicides could be used to inhibit growth of *B. cinerea.* However, it is likely that the boscalid resistant isolates could also be resistant to the FRAC 7 fungicides because they also target Sdh.

A majority of the *B. cinerea* isolates in this study contained the transposable elements, *Boty* and *Flipper*. The presence of the transposable elements can help track the population spread of *B. cinerea* in the fields. Additionally, a correlation was found between the presence of the *Boty* and *Flipper* transposons within the genome of the isolate and county source so these genetic markers can help track the migration of the plant pathogen across counties or even states.

The resistant isolates experienced differing levels of resistance. The differences could be a result of a number of reasons including overall fitness of the isolate, and the different mutations found within the genome of the isolate, including those within all the subunits of Sdh. Further sequencing of Sdh subunits could investigate if other mutations that change amino acid content are present and if those mutations influence sensitivity or resistance of the isolate to boscalid.

Future research can include analyzing the sequence of the *sdh* subunits to look for further mutations. This study only analyzed the *sdhB* sequence of 10 isolates so future studies can aim to analyze at least 50 *B. cinerea* strains, similar to Leroux et. al's 2010 study. Additionally, 5ppm is usually used as the discriminatory dose, so 5 ppm could be among the concentrations directly tested (Stockwell *et al*. 2018).

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References

Aubee C, Lieu D (2010). Environmental fate and ecological risk assessment for boscalid new use on rapeseed, including canola (seed treatment). Environmental Protection Agency. https://www3.epa.gov/pesticides/chem_search/cleared_reviews/csr_PC-128008_23-Dec-10_a.pdf. Accessed 5 August 2019.

Cenis, J. (1992). Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research,* 20(9), 2380-2380. doi: 10.1093/nar/20.9.2380

Dean, R., Kan J. A., Z. A., Hammond-Kosack, K. E., Pietro, A. D., Spanu, P.D.,…Foster, G.D. (2012). The top 10 fungal pathogens in molecular plant pathology. Mol. Plant Pathol. 13: 414-430. doi: 10.1111/j.1364-3703.2011.00783.x

Dehne H.W, Deising H.B, Gisi U, Kuck K.H, Russell P.E, Lyr H. (2010) Mode in fungicides and antifungal compounds VI. DPG Spectrum Phytomedizin. http://dpg.phytomedizin.org/fileadmin/daten/04_Verlag/02_SP/05_Reinhard/0294-sp-2011-Reinh-8.pdf. Accessed 6 August 2019.

Fernandez-Ortuño, D., Perez-Garcia, Chamorro M, Del la Peña E, De Vicente A, Tores JA (2017). Resistance to the SDHI fungicides boscalid, fluopyram, fluxapyroxad, and penthiopyrad in *Botrytis cinerea* from commercial strawberry fields in Spain. Plant Dis. 101:1306-1313. http://dx.doi.org/10.1094/PDIS-01-17-0067-RE

Fillinger, S., and Walker, A.S (2016). Chemical control and resistance management of *Botrytis* disease. In: Fillinger S, Elad Y (ed) Botrytis- the Fungus, the Pathogen and its Management in the Agricultural Systems. Springer, New York, pp 189-216.

Lalève, A., Gamet, S., Walker, A.S., Debieu D., Toquin V., and Fillinger, S. (2014). Site-directed mutagenesis of the P225, N230 and H272 residues of succinate dehydrogenase subunit B from *Botrytis cinerea* highlights different roles in enzyme activity and inhibitor binding. Environ. Microbiol. 16, 2253–2266. https://doi.org/10.1111/1462-2920.12282

Leroux, P., Gredt, M., Leroch, M., and Walker, A. (2010). Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. Appl. Environ. Microbiol. 76: 6615-6630. Retrieved June 14, 2019, from https://aem.asm.org/content/aem/76/19/6615.full.pdf

Ma, Z., and Michailides, T. J. 2005. Genetic structure of *Botrytis cinerea* populations from different host plants in California. Plant Dis. 89:1083-1089.

Myresiotis, C. K., Bardas, G. A., and Karaoglanidis, G. S. (2008). Baseline sensitivity of *Botrytis cinerea* to pyraclostrobin and boscalid and control of anilinopyrimidine- and benzimidazole- resistant strains by these fungicides. Plant Dis. 92:1427-1431.

Samuel, S., Veloukas, T., Papavasileiou, A., and Karaoglanidis, G. S. (2012) Differences in frequency of transposable elements presence in *Botrytis cinerea* populations from several hosts in Greece. Plant Dis. 96: 1286-1290. doi: 10.1094/PDIS-01-12-0103-RE.

Stockwell, V.O., Shaffer, B.T., Jones, L.A., Pscheidt, J.W. 2018. Fungicide resistance profiles of *Botrytis cinerea* isolated from berry crops in Oregon. Abstract for International Congress of Plant Pathology: Plant Health in A Global Economy; 2018 July 29-Aug 3; Boston, MA

Veloukas, T., Markoglou, A.N. and Karaoglanidis, G. S. (2013). Differential effect of *sdh*B gene mutations on the sensitivity to SDHI fungicides in *Botrytis cinerea*. Plant Dis. 97:118-122. https://doi.org/10.1094/PDIS-03-12-0322-RE

Table I. Sources and characteristics of *Botrytis cinerea* isolates from Oregon small fruits

Isolate	Year of isolation	Location (County)	Host	Boscalid EC50 (with standard deviation)	Amino acid at codon 272	Transposon d	
						Boty	*F*
DR-26-15-P6-3A	2017	Polk	Blueberry	0.47 ± 0.22	Histidine	ND^a	
LJ26-2-1	2014	Marion	Blackberry	0.21 ±0.03	Histidine	ND	
LJ171-3	2015	Lane	Blackberry	0.44 ±0.26	Histidine	ND	
LJ199-3	2015	Clackamas	Raspberry	0.77 ±0.30	Arginine	$+^b$	
LJ200-11	2015	Clackamas	Blueberry	0.43 ±0.28	Arginine	+	
NF-41-8.2	2017	Douglas	Blueberry	1.02 ±0.28	Histidine	ND	
OB-LE-2NS-1.3	2017	Washington	Blueberry	5.34 ±2.23	Arginine	+	
TS-14-33-P7-1C	2017	Polk	Blueberry	0.12 ±0.08	Histidine	ND	
UN17-VN-1-2.2	2017	Washington	Raspberry	2.14 ±1.23	Arginine	+	
.UN17-VN-4-..8.4	2017	Washington	Raspberry	34.84 ±32.26	Arginine	+	

a ND indicates that an amplicon for the *Boty* or *Flipper* transposon was not detected with PCR.
b + indicates that an amplicon for the *Boty* or *Flipper* transposon was detected with PCR.

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Table II. Primers used for *Boty*, *Flipper*, and *sdhB* amplification

Target	Primer name	Sequence	Annealing temperature	Amplicon size (bp)	Source
***sdh*B**	**IpBcBeg**	5'-CCACTCCTCCATAATGGCTGCTCTCCGC-3'	68°C	1,021	Leroux *et al*. 2010
	IpBcEnd2	5'-CTCATCAAGCCCCCTCATTGATATC-3'			
Flipper	**F300F**	5'-GCACAAAACCTACAGAAGA-3'	60° C	1,250	Ma and Michailides, 2005
	F1150	5'-ATTCGTTTCTTGGACTGTA-3'			
Boty	**BotyF4**	5'-CAGCTGCAGTATACTGGGGGA-3'	68° C	764	Ma and Michailides, 2005
	BotyR4	5'-GGTGCTCAAAGTGTTACGGGAG-3'			

Table III. Relative growth of fungal strains on media amended with different concentrations of boscalid

	Boscalid concentration (ppm)					
Isolate	0[b]	0.01	0.1	1	10	100[b]
DR-26-15-P6-3A	**100**[a]	**91**	**58**	**52**	**52**	17
LJ26-2-1	**100**	**85**	**61**	41	18	8
LJ171-3	**100**	**83**	**51**	40	19	11
LJ199-3	**100**	**120**	**110**	**88**	**78**	**74**
LJ200-11	**100**	**100**	**95**	**71**	**57**	47
NF-41-8.2	**100**	**83**	**63**	50	50	13
OB-LE-2NS-1.3	**100**	**100**	**99**	**60**	**62**	46
TS-14-33-P7-1C	**100**	**89**	**52**	39	39	11
UN17-VN-1-2.2	**100**	**100**	**91**	**76**	**76**	**51**
UN-17-VN-4-8.4	**100**	**100**	**100**	**82**	**82**	**54**

[a] 0.5% YEA medium was amended with boscalid to achieve the concentration series. Percent growth for each isolate at each concentration of boscalid was calculated through division of diameter of growth in a medium amended with boscalid by the diameter of growth in the non-amended medium. Results shown are from one experiment. A second experiment yielded similar results. Percent growth values greater than 50% are highlighted as bold, red text.

[b] 0.5% YEA medium not amended with boscalid was used as the negative control. 0.5% YEA medium amended with 100 ppm of boscalid was used as the positive control.

Table IV. The boscalid resistance classification of the 10 *B. cinerea* isolates based on percent growth at 1 ppm of boscalid amended with 0.5% Yeast Extract Agar.

Isolate	[1]Boscalid Experiment Classification
DR-26-15-P6-3A	Resistant
LJ26-2-1	Sensitive
LJ171-3	Sensitive
LJ199-3	Resistant
LJ200-11	Resistant
NF-41-8.2	Sensitive
OB-LE-2NS-1.3	Resistant
TS-14-33-P7-1C	Sensitive
UN17-VN-1-2.2	Resistant
UN17-VN-4-8.4	Resistant

[1] Isolates displaying less than 50% growth at 1ppm of boscalid as compared to 0 ppm of boscalid were classified as sensitive. Those that displayed more than or equal to 50% were classified as resistant.

Table V. Predicted partial amino acid sequence of SdhB of *Botrytis cinerea* isolates

Isolate	Boscalid sensitivity	Predicted amino acid sequence of SdhB (codon positions 240 to 290, amino acid at 272 is highlighted)
		240 250 260 270 280 290
DR-26-15-P6-3A	**Resistant**	LLOSYRWLADSRDOKKEERKAALDNSMSLYRC**H**TILNCSRTCPKGLNPGLA
LJ26-2-1	**Sensitive**	LLOSYRWLADSRDOKKEERKAALDNSMSLYRC**H**TILNCSRTCPKGLNPGLA
LJ171-3	**Sensitive**	LLOSYRWLADSRDOKKEERKAALDNSMSLYRC**H**TILNCSRTCPKGLNPGLA
LJ199-3	**Resistant**	LLOSYRWLADSRDOKKEERKAALDNSMSLYRC**R**TILNCSRTCPKGLNPGLA
LJ200-11	**Resistant**	LLOSYRWLADSRDOKKEERKAALDNSMSLYRC**R**TILNCSRTCPKGLNPGLA
NF-41-8.2	**Sensitive**	LLOSYRWLADSRDOKKEERKAALDNSMSLYRC**H**TILNCSRTCPKG
OB-LE-2NS-1.3	**Resistant**	LLOSYRWLADSRDOKKEERKAALDNSMSLYRC**R**TILNCSRTCPKGLNPGLA
TS-14-33-P7-1C	**Sensitive**	LLOSYRWLADSRDOKKEERKAALDNSMSLYRC**H**TILNCSRTCPKGLNPGLA
UN17-VN-1-2.2	**Resistant**	LLOSYRWLADSRDOKKEERKAALDNSMSLYRC**R**TILNCSRTCPKGLNPGLA
UN17-VN-4-8.4	**Resistant**	LLOSYRWLADSRDOKKEERKAALDNSMSLYRC**R**TILNCSRTCPKGLNPGLA