Characterization and Pathogenicity of Stem Blight Complex Isolates Associated with Stem Blight Disease on *Vaccinium* Species

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Abstract. Species of *Botryosphaeria* and *Neofusicoccum* are major pathogens of blueberry worldwide. Accurate identification of these species is essential for developing effective management practices. A multigene sequencing strategy was used to distinguish between six isolates of stem blight pathogens collected from two different regions of the United States. The temperature growth study revealed that the optimal temperature for growth of five of the tested isolates ranged from 25 to 30 °C, although no significant difference was detected for the growth of *Neofusicoccum* spp. isolate SD16-86 at 20, 25, 30, and 35 °C. In vitro fungicide assays showed four fungicides, cyprodinil + fludioxonil, propiconazole, pyraclostrobin + boscalid, and azoxystrobin, were effective against the tested isolates with isolate SD16-86 being less sensitive compared with the other isolates. In a detached stem assay, none of 39 blueberry accessions displayed immunity or a high level of resistance to the two tested isolates, and no significant difference in lesion length was detected among the seven tested *Vaccinium* species inoculated with the two isolates.

Blueberries rank as the second most important berry crop in North America with a total area of 96,869 ha (Strik, 2006). As blueberry acreage increases, pathogen diversity and diseases become a more important issue. Stem blight, caused by the species complex of *Botryosphaeria* Moug.:Fr and *Neofusicoccum*, Crous, Slippers and A. J. L. Phillips, is considered one of the most common diseases limiting the establishment of blueberry plantings in the southeastern United States (Ballington et al., 1993; Wright and Harmon, 2010). In addition to blueberry, these causal fungi infect other woody plants, thus increasing the likelihood that field-grown blueberry plants will be exposed to inoculum of these pathogens. Species of

Botryosphaeria and *Neofusicoccum* infect blueberry plants through wounds and spread from infected plants to healthy plants by wind, rain, and field equipment (Milholland, 1972).

Disease symptoms include reddening and drying of leaves, wilting of infected shoots, and plant death as the pathogen spreads (Polashock et al., 2017; Wright and Harmon, 2010). Incidence of stem blight has been increasing in the United States, and the disease has been reported in many other countries, including Mexico (Boyzo-Marin et al., 2016), New Zealand (Tennakoon et al., 2018), and China (Yu et al., 2012). Various management strategies have been recommended to reduce the severity of the disease, including irrigation management (Creswell and Milholland, 1988), use of resistant cultivars and clean planting stock, and selective pruning of infected parts (Weaver, 1978). Information about fungicide sensitivity could be used to reduce the severity of the disease when applied after pruning or injury to plants. Efficacy of fungicides to control the growth of *B. dothidea* was determined in a previous study using a detached stem assay. Stems treated with cyprodinil, fludioxonil, and pyraclostrobin developed lesions shorter than those of nontreated control (Smith and Miller-Butler, 2017b).

For U.S. producers to maintain competitiveness, new and highly productive cultivars possessing enhanced tolerance to biotic and abiotic stresses are needed. However, identifying the causal pathogen(s) is critical before developing resistant cultivars as well as implementing effective management and quarantine strategies. Breeding for disease resistance relies on accurate identification of the causal pathogens before incorporating genes for resistance. Resistance of certain half-high and lowbush blueberry cultivars to *Botryosphaeria* has been reported (Polashock and Kramer, 2006). Southern highbush blueberry (species complex between *V. corymbosum* L. $2n = 4x = 48$ and *V. darrowii* Camp $2n = 2x = 24$) breeders

Fig. 1. Neighbor-joining tree based on concatenated ITS and partial EF1- α sequence data. Bootstrap values for 1000 iterations are shown at the branch junctions. Data for vouchered specimens (*Neofusicoccum kwambonambiense* isolate CBS123639, *N. umdonicola* isolate CBS123645, *N. ribis* isolate CBS115475, *N. parvum* isolate ATCC5891, *Botryosphaeria dothidea* isolate CBS115476, and *Lasiodiplodia theobromae* isolate CBS16496) were downloaded from GenBank. Bar indicates nucleotide substitutions.

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have used various native *Vaccinium* species in crosses to incorporate genes for adaptation; however, little is known about *Neofusicoccum* species identification and the responses of native *Vaccinium* species to infection. Morphological differentiation between different species of *Botryosphaeria* and *Neofusicoccum* may not be accurate because isolate pigmentation varies with temperature (Wright and Harmon, 2010). Differentiation based on multigene sequence analyses has proven to be a more reliable method of differentiation between species (Wright and Harmon, 2010). The objectives of our research were to 1) characterize six isolates of stem blight pathogens based on comparison of the DNA sequences of internal transcribed spacer (ITS) and elongation factor (EF1- α), 2) determine the optimal temperature for the growth of these isolates, 3) characterize the efficacy of several fungicides in controlling the growth of these isolates, and 4) conduct pathogenicity tests on 39 accessions from different *Vaccinium* species.

Materials and Methods

Fungal isolates and molecular characterization. Stems were collected from symptomatic southern highbush blueberry cultivars Rebel and Pearl River and used to obtain isolates labeled as Rebel and Pearl River respectively following a protocol described by Smith (2009). In addition, four isolates, SD14-121, SD14-135, SD16-86, and SD16-175, were obtained from infected blueberry samples collected from the P.E. Marucci Center for Blueberry and Cranberry Research, Chatsworth, NJ, as described by Polashock and Kramer (2006). Genomic DNA was extracted from the mycelium of

each isolate following a modified CTAB method described by Babiker et al. (2012). Two sets of primers were used to amplify the internal transcribed spacer (ITS) of rDNA and a partial sequence of the elongation factor (EF1- α) from the six isolates following the protocol described by White et al. (1990) and Carbone and Kohn (1999), respectively. Polymerase chain reaction products were analyzed in 1.5% agarose gel stained with ethidium bromide, and the desired amplification products from the six isolates were purified using Zymoclean gel DNA recovery kit (Zymo Research, Irvine, CA). Purified fragments were cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced using the BigDye-Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI3500 Genetic Analyzer (Applied Biosystems). The ITS and EF1- α sequences were concatenated and compared with representative voucher sequences of *Neofusicoccum* spp. and *Botryosphaeria dothidea* downloaded from GenBank. These voucher data represent sequences from *N. kwambonambiense* isolate CBS123639, *N. umdonicola* isolate CBS123645, *N. ribis* isolate CBS115475, *N. parvum* isolate ATCC5891, *B. dothidea* isolate CBS115476, and the outgroup *Lasiodiplodia theobromae* isolate CBS16496. Alignment was done using Muscle in Mega6 (Tamura et al., 2013). A bootstrapped neighbor-joining tree was generated using Mega6.

Temperature growth study. To determine the optimal temperature for the growth in culture of five isolates, SD14-121, SD14-135, SD16-86, SD16-175, and Rebel, 5.5-mm plugs from each isolate were placed in the center of 10-cm diameter petri dishes containing potato dextrose agar (PDA). For each isolate, two replicates were incubated in four

growth chambers maintained at 20, 25, 30, and 35 °C in the dark. Colony diameters were measured after 2 and 5 d.

In vitro fungicide sensitivity tests. Isolates SD14-121, SD14-135, SD16-86, SD16-175, and Rebel were tested for their sensitivity to four fungicides registered for blueberry disease management; azoxystrobin (Abound, Syngenta Crop Protection, Greensboro, NC), pyraclostrobin + boscalid (Pristine, BASF Corporation, Research Triangle Park, NC), propiconazole (Orbit, Syngenta Crop Protection, Greensboro, NC), and cyprodinil + fludioxonil (Switch, Syngenta Crop Protection, Greensboro, NC). Stock solutions of propiconazole, pyraclostrobin + boscalid, azoxystrobin, and cyprodinil + fludioxonil at concentration of 41.8%, 12.8% + 25.2%, 22.9%, and 37.5% + 25% a.i., respectively, were added into autoclaved and cooled PDA medium. Approximately 25 mL of the amended PDA were poured into 10-cm diameter petri dishes to obtain $\approx$ 5-mm-thick layer of agar. A 5.5-mm plug from each isolate was placed in the center of a petri dish plate containing PDA amended with each fungicide. After 7 d, colony diameter was measured. The percentage of mycelial growth inhibition (MGI) was calculated from the colony diameter in fungicide amended PDA (FA) and nonamended PDA (NA) using the formula

$$\text{MGI (\%)} = [(NA - FA)/NA] \times 100$$

Pathogenicity tests. On the basis of results of the temperature growth study, the two fastest growing isolates of the stem blight complex, Rebel and SD16-86, were selected and cultured on PDA. Mycelial plugs from 7-day-old cultures were used to screen 39 accessions representing seven *Vaccinium* species: *V. darrowii* Camp ($2n = 2x = 24$),

Table 1. Analysis of variance probability levels for colony diameter of five stem blight complex isolates at four temperatures after 5 d growth on potato dextrose agar.

Source	df	Mean square	F	Pr > F
Isolate	4	291.54	61.78	<0.0001
Temperature	3	1562.97	331.2	<0.0001
Replicate	1	16.20	3.43	0.069
Isolate * temperature	12	442.57	93.78	<0.0001

Table 2. Mean colony diameter of five stem blight complex isolates after 5 d growth on potato dextrose agar across four temperatures (20, 25, 30, and 35 °C).

Isolate	Colony diam (mm) ²
SD16-86	85.0 a
Rebel	75.1 b
SD16-175	75.4 b
SD14-121	76.0 b
SD14-135	75.4 b
LSD ($P = 0.05$)	1.54

²Means followed by different letters are significantly different at 5% level of probability according to Fisher's protected least significant difference (LSD) test.

Fig. 2. Colony diameter of five stem blight complex isolates grown on potato dextrose agar incubated at four temperatures after 5 d. Bars indicate $\pm$ SE of the mean. *Temperature that significantly caused reduction in colony diameter of the tested isolate compared with other temperatures (based on least square mean test at $P = 0.05$).

V. elliptii Chapm. ($2n = 2x = 24$), *V. stamineum* Lam. ($2n = 4x = 48$), *V. arboreum* ($2n = 2x = 24$), *V. pallidum* Ait. ($2n = 2x = 24$), *V. corymbosum* ($2n = 4x = 48$), and *V. virgatum* Aiton. ($2n = 6x = 72$), using a detached stem assay (Smith, 2009). Briefly, succulent, partially hardened-off stems with similar diameters and 15 to 20 cm in length were collected from the 39 accessions grown in pots maintained in a greenhouse using pruning shears. All leaves except the terminal three were excised with a razor blade, and the stems were immersed in a beaker with a 10%

Table 3. Analysis of variance probability levels for growth inhibition of five stem blight complex isolates in potato dextrose agar media amended with four fungicides after 7 d.

Source	df	Mean square	F	Pr > F
Isolate	4	1,428.17	196.04	<0.0001
Fungicide	3	11,655.83	1,599.92	<0.0001
Replicate	2	14.16	1.94	0.1685
Isolate * fungicide	12	471.89	64.77	<0.0001

Means were calculated from three replicates per isolate across two experimental runs.

Table 4. Impact of four fungicides on percentage of growth inhibition of five stem blight complex isolates after 5 d on fungicide amended agar.

Fungicide	Percent of inhibition ^a
Azoxystrobin	35.82 d
Pyraclostrobin + boscalid	80.39 c
Propiconazole	82.55 b
Cyprodinil + fludioxonil	88.06 a
LSD ($P = 0.05$)	1.71

^aMeans followed by different letters are significantly different at 5% level of probability according to Fisher's protected least significant difference (LSD) test.

solution of sodium hypochlorite for 15 min followed by dipping in 75% ethanol for 15 s and three rinses in sterile deionized water. A small section (2×10 mm) of the bark from each stem was removed using a sterile scalpel. Mycelial plugs taken from the 7-day-old cultures using a 5.5-mm-diameter sterile cork borer were sealed to the wounded sections using a strip of parafilm. Control stems were treated similarly except that they were mock inoculated with agar plugs from sterile PDA. Stems were inserted into 12-cm test tubes containing 25-mm-deep sterile distilled water. Nine stems from each accession were inoculated with each of the two isolates. The experiment was repeated twice. All inoculated and noninoculated stems were incubated in dew chambers maintained at 25 °C and ~80% relative humidity with continuous light. After 7 d, the parafilm was removed from each stem and lesion length was measured.

Statistical analysis. Analysis of variance was calculated using proc GLM in SAS (version 9.4; SAS Institute, Cary, NC). All experiments were conducted twice and means of isolates, temperature, and fungicides were compared using Fisher's protected least significant difference at $P = 0.05$. Means of temperature $\times$ isolate and accession $\times$ isolate interactions were compared using least square means at $P = 0.05$.

Results

Molecular characterization. The neighbor joining tree generated from the sequence data suggested that the Pearl River, Rebel, SD16-86, and SD16-175 isolates are *Neofusicoccum* spp., whereas SD14-121 and SD14-135 are *B. dothidea* (Fig. 1).

Temperature growth study. The temperature and isolate had significant effects on

colony diameter (Table 1). The 2- and 5-day measurements indicated that the maximum colony diameter for the five isolates occurred at 25 and 30 °C ($P < 0.05$). Isolate SD16-86 displayed a significantly faster rate of growth compared with the other isolates across the four temperatures ($P < 0.05$; Table 2). Significant interaction between temperature and isolate ($P < 0.05$; Table 1) indicated that *Neofusicoccum* spp. isolate SD16-86 is adapted to a wide range of temperatures because there was no significant difference between growth rate of isolate SD16-86 at the four tested temperatures ($P < 0.05$; Fig. 2). Except for *Neofusicoccum* spp. isolate SD16-86, 35 °C significantly inhibited the growth of isolates SD14-121, SD14-135, SD16-175, and Rebel ($P < 0.05$; Fig. 2). Furthermore, *B. dothidea* isolates SD14-121 and SD14-135 grew slower at 20 °C compared with 25, 30, and 35 °C. Growth of *Neofusicoccum* spp. isolates Rebel and SD16-175 were significantly slower at 35 °C than at 20, 25, and 30 °C ($P < 0.05$; Fig. 2).

In vitro fungicide sensitivity tests. Fungicides, isolates, and fungicide $\times$ isolate interaction had significant effects on mycelium growth ($P < 0.05$; Table 3). Cyprodinil + fludioxonil had the highest percentage of growth inhibition followed by propiconazole, pyraclostrobin + boscalid, and azoxystrobin respectively ($P < 0.05$). In addition, growth of *Neofusicoccum* spp. isolate SD16-86 displayed significantly greater inhibition followed by isolates Rebel, SD16-175, SD14-135, and SD14-121, respectively ($P < 0.05$; Table 4). However, the significant fungicide $\times$ isolate interaction indicated that the strength of the mycelial growth inhibition depends on the isolate ($P < 0.05$; Fig. 3). When tested against isolate SD14-135, pyraclostrobin + boscalid caused significant mycelial growth inhibition as compared with propiconazole ($P < 0.05$; Fig. 3).

Pathogenicity tests. All 39 blueberry accessions inoculated with *Neofusicoccum* spp. isolates SD16-86 and Rebel developed typical stem blight lesions that were significantly longer than lesions from the non-inoculated control. None of the accessions exhibited immunity ($P < 0.05$; Fig. 4). There was a significant effect of isolate and accession on lesion length ($P < 0.05$; Table 5). In the two trials, accessions inoculated with isolate Rebel produced significantly longer lesions than those inoculated with isolate SD16-86 ($P < 0.05$; Table 6). Neither *Vaccinium* species nor accession $\times$ isolate interaction had a significant effect on lesion length.

Discussion

A recent survey of blueberry growers in the United States and Canada indicated that stem blight is among the more important blueberry diseases (Gallardo et al., 2018). Accelerating the evaluation process and increasing the accuracy of selection are necessary for introgression of disease resistance

Fig. 3. Percentage of growth inhibition of five stem blight complex isolates grown on potato dextrose agar amended with four fungicides after 7 d. Bars indicate $\pm$ SE of the mean. *Fungicide that significantly caused growth inhibition of the tested isolate compared with other tested fungicides (based on least square mean test at $P = 0.05$).

Fig. 4. Mean lesion length displayed by detached stems of 39 accessions of seven *Vaccinium* species inoculated with two isolates of stem blight complex in (A) 2017 and (B) 2018. Vc= *Vaccinium corymbosum*, Vv = *V. virgatum*, Ve = *Vaccinium elliotii*, Vd = *V. darrowii*, Va = *V. arboreum*, Vs = *V. stamineum*, and Vp = *V. pallidum*. Bars indicate $\pm$ SE of the mean.

genes into widely adapted blueberry cultivars. Breeding for disease resistance relies on finding sources of resistance within cultivated and wild species using the most common and diverse isolates in the region under optimal conditions. Previous studies used in vitro assays to determine the optimal temperature for the growth of stem blight pathogens. Our temperature growth study revealed that

the optimal temperature for the growth of isolates Rebel and SD16-175 ranged from 20 to 30 °C, whereas for SD14-121 and SD14-135 ranged from 25 to 30 °C. This variation could be attributed to the fact that isolates SD14-121 and SD14-135 are likely *B. dothidea*, whereas the others are *Neofusicoccum* spp. This finding is consistent with those of Smith and Miller-Butler

(2017a), who reported optimal growth of their *B. dothidea* isolates occurred at 25, 30, and 35 °C, and with Wright and Harmon (2010), who reported that the optimal temperature for the growth of *B. dothidea*, *Lasiodiplodia theobromae*, *N. parvum*, and *N. ribis* isolates is 30 °C. In our study, isolate SD16-86, obtained from blueberries in New Jersey, grew over a range of

Table 5. Analysis of variance of average lesion length (cm) of detached stems of 39 accessions from seven *Vaccinium* species inoculated with two stem blight complex isolates, SD16-86 and Rebel.

Source	df	Mean	F	Pr > F
Year	1	245.87	10.12	0.0015
Accession	38	106.95	4.4	<0.0001
Isolate	1	1,190.38	49.02	<0.0001
Species	6	2.78	0.11	0.8918
Replicate	3	87.68	3.61	0.0131
Accession * isolate	38	28.66	1.18	0.2138

Table 6. Mean lesion length following inoculation of detached stems of 39 accessions from seven *Vaccinium* species with two stem blight complex isolates in 2017 and 2018.

Isolate	Lesion length (cm) ^c
Rebel	9.81 a
SD16-86	7.30 b
LSD (<i>P</i> = 0.05)	0.63

^cMeans followed by different letters are significantly different at 5% level of probability according to Fisher's protected least significant difference (LSD) test.

temperatures (20 to 35 °C), whereas colony diameter of cultures of isolate Rebel, obtained from symptomatic stems collected from Mississippi, significantly decreased at 35 °C. Diameter of fungal colonies grown on plates may overestimate the survival potential of isolates; therefore, further field research is needed to confirm this finding.

Results from the fungicide sensitivity tests showed that growing stem blight complex isolates in PDA amended with either of cyprodinil + fludioxonil, propiconazole, pyraclostrobin + boscalid, and azoxystrobin inhibited mycelial growth. Growth inhibition on Switch (cyprodinil + fludioxonil) amended plates was significantly greater than that of plates amended with the other tested fungicides. This finding is consistent with Smith and Miller-Butler (2017b), who reported a significant reduction in stem blight lesion length of detached blueberry stems treated with cyprodinil + fludioxonil. This result could be attributed to the fact that Switch contains two fungicides with different modes of action. Cyprodinil is an anilino-pyrimidine that inhibits the methionine biosynthesis pathway, whereas fludioxonil stimulates the synthesis of glycerol, which inhibits fungal mycelial growth (Leroux, 1997). Although Switch (cyprodinil + fludioxonil) is highly effective against tested isolates, the fungicide should be tested according to the fungicide resistance action committee guidelines. Further research is needed to test the efficacy of these fungicides in the field.

Genetic resistance is the most effective means of disease control. Although there are commercial southern highbush blueberry cultivars with moderate levels of resistance to *B. dothidea* (Smith, 2009), responses of southern highbush blueberry cultivars and native *Vaccinium* species to isolates of the stem blight complex have not been investigated. In

this study, the detached stem assay showed the reaction of different accessions from seven *Vaccinium* species varied across isolates, and none of the tested accessions displayed immunity to either of the two tested isolates. This finding is consistent with Tennakoon et al. (2018), who found pathogenicity of *Neofusicoccum* differed significantly between species and isolates within a species. Further, no significant difference in lesion length was detected between the different *Vaccinium* species, suggesting that the host range for stem blight isolates is not limited to cultivated blueberry (*V. corymbosum* and *V. virgatum*). The detached stem assay showed that accessions from the native species, *V. elliotii* and *V. arboreum*, had shorter lesions compared with accessions from *V. corymbosum* and *V. virgatum*. Results from this study are consistent with Ballington et al. (1993), who found *V. elliotii* and *V. arboreum* to be resistant to *B. dothidea*.

The outcome of this study indicates that *Neofusicoccum* spp. isolate SD16-86 was well adapted to a range of temperatures extending from 20 to 35 °C and was less sensitive to fungicides compared with other tested isolates. However, isolate Rebel was significantly more aggressive than isolate SD16-86. Three fungicides, cyprodinil + fludioxonil, propiconazole, and pyraclostrobin + boscalid, were effective in vitro assays against tested isolates of the stem blight complex. Because an association between in vitro fungicide assays and field application was not evaluated in this study, future field research is needed to confirm the efficacy of these fungicides against stem blight incidence. Furthermore, no significant difference in lesion length was detected between the different *Vaccinium* species, and none of the tested accessions was immune or highly resistance to the tested isolates.

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