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Screening Southern Highbush Blueberry Genotypes for *Botryosphaeria* Stem Blight

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Abstract

Stem blight and dieback are caused by a complex of fungi in the Botryosphaeriaceae (Bot) and are among the most damaging fungal pathogens affecting southern highbush blueberries (SHB; *Vaccinium corymbosum* L. hybrids) in the southeastern U.S. Currently, cultural practices do not effectively manage stem blight and dieback; therefore, resistance breeding offers the most promising method of control. A stem inoculation technique was used to screen SHB genotypes for resistance to stem blight. Two inoculum sources commonly found in southeastern production regions were tested on six cultivars to determine whether an individual isolate could be used for disease screening. There were significant differences between cultivar susceptibility and inoculum sources ($P < 0.05$), but there was no significant interaction between cultivar and inoculum source ($P = 0.14$), indicating a single inoculum source could be used in further experiments. Un-rooted softwood cuttings collected from nine SHB cultivars and advanced selections to be used as parents in the University of Florida (UF) breeding program were tested in five successive inoculations. Genotype ($P < 0.0001$) and experiment ($P < 0.0001$) main effects, as well as the genotype by experiment interaction ($P = 0.0003$) were significantly different, indicating a lack of repeatability for this screening method.

Blueberry production in Florida has increased in the past decade from approximately 650 to just over 1,800 ha, with a farm gate value in 2012 of nearly $70 million (USDA, 2013). To market fresh fruit in the high-value period of mid-March through April, production is now almost exclusively of SHB cultivars. The most economically damaging fungal disease in Florida SHB production is stem blight, which results in yield reduction and premature plant mortality (Lyrene, 2008). Stem blight in SHB is caused by a complex of Bot fungi including *Botryosphaeria dothidea, Lasiodiplodia theobromae,* and *Neofusicoccum ribis* (Wright and Harmon, 2010).

Bot fungi infect current season blueberry growth, and basipetal movement of the pathogen in the vasculature can progress rapidly (Witcher and Clayton, 1962). Stem blight symptoms typically include rapid wilting and reddening of leaves on affected branches (Milholland, 1972). In severe cases, infection progresses into the crown of the blueberry plant and results in systemic branch dieback, which eventually kills the plant. Fungicide applications (Cline and Milholland, 1992; Smith, 2009), optimizing irrigation practices (Creswell and Milholland, 1988; Michailides and Morgan, 1992), and aggressive pruning (Weaver, 1978) for the control of stem blight increases production costs and do not adequately manage the disease (Cline et al., 1993).

With the lack of effective chemical and cultural control methods, resistance breeding is the most promising method for management of stem blight in SHB. However, difficulties in assigning a heritability estimate in previous studies have been attributed to differences in isolate virulence (Buckley, 1990), and plant stress (Smith, 2004). Cultivar

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variation for stem blight susceptibility has been identified by *in vitro* and with cut stem inoculations (Polashock and Kramer 2006; Smith, 2004, 2006, 2009), but did not evaluate the low-chill SHB germplasm used in the UF blueberry breeding program or inoculation with disease isolates commonly found in Florida (Wright and Harmon, 2010).

Our objectives were to determine whether genotype response to pathogen development differed based on inoculum source, assess the repeatability of un-rooted stem cutting inoculation as a screening assay, and to screen SHB genotypes for susceptibility to stem blight prior to use in crossing.

Materials and Methods

Differential Response to Pathogen Isolates. In the summer of 2009, plants of the cultivars 'Emerald', 'Jewel', 'Misty', 'Primadonna', 'Snowchaser', and 'Springhigh' were obtained from a commercial blueberry nursery (Fall Creek Farm & Nursery, Inc. Lowell, OR). Although complete resistance to stem blight has not been observed among SHB genotypes, those included in this experiment have shown a range of field susceptibility. 'Emerald' and 'Springhigh' have a high degree of field tolerance, while 'Snowchaser' and 'Misty' often show stem blight symptoms after plants have been exposed to biotic or abiotic stress. The vegetative plants were sheared to a uniform height of 35 cm prior to inoculation. Isolates had been collected and stored on filter paper at 4°C as part of a previous study (Wright and Harmon, 2010). Isolates were revived by placing infested filter paper strips onto plates of V8 agar (15.0 g agar, 200 mL V8 juice (CSC Brands LP), 2.0 g $CaCO_3$, 0.01 mg of rifampicin and 0.25 g of ampicillin sodium salt in 1 L) at 25°C (Wright and Harmon, 2010). Plugs (8 mm diam.) were taken from the margins of colonies of *L. theobromae* and *N. ribis* isolates, and from sterile V8 agar plates as controls. The plugs were placed directly on the cut end of a SHB stem and secured with Parafilm (Pechiney Plastic Packaging Co. Chicago, IL). Five plants of each cultivar were inoculated separately with *L. theobromae* isolate MixFC6 or *N. ribis* isolate UF0440. Plants were grown in a greenhouse with temperatures ranging between 20 to 30°C; environmental conditions remained unchanged for the duration of the experiments. The experiment was a randomized complete block design (RCBD) and was repeated twice. Developing lesions were measured twice weekly for three weeks, to calculate the area under the disease progress curve (AUDPC). Statistical significance was calculated using analysis of variance with a general liner model and a Waller Duncan *k*-ratio *t*-test ($k = 100$) separated cultivar and inoculum effects (SAS 9.2, SAS Institute, Cary, NC).

Un-rooted Softwood Cutting Inoculations. Four cultivars ('Snowchaser', 'Sweetcrisp', 'Springhigh', and 'Windsor') were used along with five selections from the UF SHB breeding program (FL98-325, FL06-372, FL06-382, FL06-483, and FL06-559). All genotypes were assumed to be susceptible to stem blight. Approximately 15 clonal softwood cuttings, between nine and 16 cm in length were taken from a location mid-way down a non-woody cane (leafy shoot) and were collected biweekly from 26 August to 11 November, 2009. The cuttings ranged from approximately 5 to 8 mm in diameter. Shears were sterilized with 95% ethanol between each genotype and after every four to five cuts. The cuttings were moistened with tap water and were placed on ice for 24 h. After 24 h, the cuttings were washed with 10% bleach for one min prior to planting in 3.75 L pots that had previously been disinfested with 10% bleach for one min. The un-rooted cuttings were planted in 100% sphagnum moss moistened with tap water and arranged in a RCBD. Nine cuttings (one per accession) were placed in each pot and remained under a misting system for 24 h prior to inoculation.

After 24 h, each cutting was trimmed so that a section of the stem with the top two to three leaves was removed. After trim-

Table 1. Analysis of variance for differential response to stem blight inoculum source. The experiment was a 6 x 2 x 2 factorial design, with six cultivars ('Jewel', 'Snowchaser', 'Misty', 'Emerald', 'Primadonna', and 'Springhigh'), two inoculum sources (*Lasiodiplodia theobromae* isolate MixFC6 and *Neofusicoccum ribis* isolate UF0440, and two replicate experiments.

Source	Degrees of Freedom	Type III Sum of Squares	Mean Square	F Value	Prob. > F
Cultivar	5	22850.0202	4570.0040	5.65	0.0001
Experiment	1	1460.2173	1460.2173	1.81	0.1821
Replication	4	4100.8056	1025.2014	1.27	0.2878
Inoculum Source	1	4602.6818	4602.6818	5.69	0.0189
Cultivar * Inoculum Source	5	6908.7104	1381.7421	1.71	0.1395

ming, cuttings from nine pots were inoculated using the method described previously with the following modifications to screen larger amounts of plant material. Only cultures of *N. ribis* UF0440 which were ground in a blender for 30 s with 250 mL of sterile distilled water (sdw) were used. Mock inoculum controls consisted of three sterile V8 agar plates blended with 150 mL of sdw. Cuttings were sprayed with inoculum until the leaves were dripping. A sterile paper towel moistened with sdw was placed on top of the inoculated cuttings. Subsequently, all plant material was bagged using sealable plastic bags and placed in a 25°C incubator (Percival Scientific, Perry IA) receiving 12 h of light (GE Chroma 50, 20 W lights) per day. After one week, the sterile paper towel covering the inoculation site was removed and lesion lengths were measured twice a week for two weeks. During this period, all pots remained bagged to ensure cuttings remained in a moist environment. Developing lesions were measured twice weekly for three weeks to calculate AUDPC. The experiment was repeated five times sequentially with nine replications per experimental date. Analysis of variance using a general linear model (SAS 9.2, SAS Institute, Cary, NC) was used for analysis, and genotype means were separated using a Waller Duncan *k*-ratio *t*-test ($k = 100$).

Results and Discussion

In a preliminary study, isolates of *Botryosphaeria dothidea*, *L. theobromae*, and *N. ribis* were inoculated onto a single blueberry cultivar; *B. dothidea* was the least virulent of the three pathogens (Wright and Harmon, 2009). Therefore, isolates of *L. theobromae* and *N. ribis* were evaluated in the present study on a group of cultivars to determine whether susceptibility to the two isolates differed. Both cultivar and inoculum source were significant factors (Table 1), but there was no significant interaction between cultivar and inoculum source (Table 1). For all cultivars except 'Springhigh', *N. ribis* resulted in longer lesion lengths after three weeks. Based on these results, a single virulent isolate of *N. ribis* was used for all inoculation experiments. Subsequently, *N. ribis* was identified as the predominant Bot species present in sampling from five southeastern states (Wright and Harmon, 2010).

Softwood cuttings were taken from the cultivars 'Snowchaser', 'Springhigh', 'Sweetcrisp', and 'Windsor', and the breeding selections FL98-325, FL06-372, FL06-382, FL06-483, and FL06-559. Nine replicates of each genotype were inoculated with an isolate of *N. ribis* for five successive experiments from late August through November, 2009. Genotype and experiment main effects were significantly different ($P < 0.0001$), and the genotype by experiment interaction was also significant ($P = 0.0003$). Therefore, cultivar differences in AUDPC are shown for each experiment separately in Table 2.

Results from this experiment were similar

Table 2. Comparisons of southern highbush blueberry genotypes after inoculation with the *Neofusicoccum ribis* isolate UF0440. Area under the disease progress curve (AUDPC) calculated from lesion length measurements on unrooted softwood cuttings. Five experiments using the same plant material and isolate were conducted sequentially in August through November, 2009.

	AUDPC				
Genotype	Expt. 1[z]	Expt. 2	Expt. 3	Expt. 4	Expt. 5
FL06-483	47.94 a	18.68 ab	31.42 a	55.19 abc	30.44 a
FL98-325	40.71 a	17.30 ab	30.83 a	65.68 ab	20.62 ab
FL06-559	22.96 b	29.79 ab	40.17 a	87.28 a	22.19 ab
FL06-382	21.76 b	12.18 b	42.99 a	25.22 cd	18.47 ab
Sweetcrisp	21.21 b	24.99 ab	28.84 a	19.63 d	15.06 b
Snowchaser	20.74 b	12.99 ab	8.43 a	36.10 bcd	14.76 b
Windsor	19.29 b	10.72 b	18.64 a	16.75 d	16.46 b
Springhigh	18.23 b	20.97 ab	34.44 a	23.50 cd	12.12 b
FL06-372	10.30 b	37.36 a	12.69 a	36.31 bcd	20.51 ab

[z]Mean separation by Waller-Duncan *K*-ratio t-test ($K = 100$). Means with the same letter within each column are not significantly different.

to previous studies; cultivars could be ranked for susceptibility to Bot pathogens based on lesion length (Cline et al., 1993; Creswell and Milholland, 1987; Polashock and Kramer, 2006; Smith, 2004). However, as the significant genotype by experiment interaction indicated, ranking of genotypes was not consistent. Some general trends were evident, such as the cultivar 'Windsor' consistently having among the smallest lesion lengths, while selections FL06-483 and FL98-325 have significantly larger lesion lengths. Most un-inoculated control cuttings were asymptomatic and appeared healthy after two weeks with no visible signs of wilting or drying. However, stem blight symptoms were apparent on up to 24% of the un-inoculated softwood cuttings for some genotypes over the course of the experiments, indicating the potential for latent infections to confound screening for resistance. There appeared to be no pattern to which genotype had latent infections present.

Neofusicoccum ribis is an opportunistic pathogen that invades wounds, and latently colonizes healthy tissue such as petioles, stems, and seed (Denman et al., 2003; Slippers and Wingfield, 2007; Swart et al., 2000). When a host becomes stressed due to abiotic or biotic factors, the infection cycle is activated (Old et al., 1990; Schoeneweiss, 1981; Slippers and Wingfield, 2007). Fungi in the Botryosphaeriaceae were previously recovered from un-inoculated genotypes used in a SHB resistance screening study (Wright, 2008). Latent infection present in plant material used for inoculation may have affected the repeatability between trials. After two weeks, stem lesions were visible on some uninoculated control treatments for all experiments; however, cuttings taken in September had higher percentages of stem blight incidence when compared to the cuttings collected in November. In Florida, conidia production of *L. theobromae* was observed most frequently during late October and early November in 2007 (Wright and Harmon, 2010). The increased presence of natural Bot inoculum in September and October in contrast to previously reported results could be due to yearly environmental fluctuations. Regardless, the presence of Bot prior to inoculation likely contributed to the discrepancies between repeated trials as lesions developing from latent infections increased in size and coalesced with lesions developing from inoculation. Although we were able to quantify susceptibility to *N. ribis* in the SHB genotypes evaluated, further assay development will be necessary for a repeatable assay to screen multiple genotypes using the inoculum sources present in Florida.

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