Screening for Susceptibility to Anthracnose Stem Lesions in Southern Highbush Blueberry

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Additional index words. *Colletotrichum*, *Vaccinium corymbosum*, Florida

Abstract. ‘Flicker’ is a southern highbush blueberry (SHB, *Vaccinium corymbosum*) cultivar frequently selected by growers in Central and South Florida. In 2014, several growers in Central Florida experienced issues with anthracnose stem lesions and twig dieback on ‘Flicker’, resulting in a reduction in new plantings and the removal of many existing plantings. The objective of this study was to determine the level of anthracnose susceptibility of certain commercially available SHB cultivars, which information can be used to limit further use of susceptible cultivars in the University of Florida blueberry breeding program. The screening was performed using a spray inoculation of a virulent *Colletotrichum gloeosporioides* isolate onto whole *V. corymbosum* plants, followed by measurement of incidence and severity of disease over time. In repeated experiments, ‘Flicker’ and two other cultivars had a significantly higher mean number of lesions and area under the disease progress curve (AUDPC) than any other tested cultivar, and in both experiments, the observed lesions were similar in many respects to those previously reported on northern highbush blueberry (also *V. corymbosum*). Although the results of these experiments may ultimately indicate that Flicker has a unique genetic susceptibility to this form of anthracnose among SHB cultivars commercially grown in Florida, screening of additional cultivars must be performed for confirmation.

Anthracnose is a group of diseases incited by fungal pathogens including those in the genus *Colletotrichum*. *Colletotrichum* is distributed primarily in tropical and subtropical regions, although it can also be found in more temperate regions (Cannon et al., 2012). The disease is characterized by dark, sunken, subcircular, or angular necrotic lesions, as shown in Fig. 1, with salmon or pink conidial masses erupting from the lesions in later stages. Lesions typically enlarge, coalesce, and can result in significant dieback (Freeman et al., 1998; Jeffries et al., 1990).

Anthracnose is typically observed as fruit rots, leaf spots, or stem lesions. Stem lesions can be incited by different species of *Colletotrichum*, and many crops grown throughout the world are susceptible to one or more species. Certain *Colletotrichum* species have been found to cause stem lesion anthracnose on cassava (Fokunang et al., 2002), mango (Gupta et al., 2015), and dragon fruit (Vijaya et al., 2015), all grown primarily in tropical regions.

There have been a few reported incidences of anthracnose stem lesions on blueberry. Kim et al. (2009) reported *C. gloeosporioides* infecting stems on highbush blueberry in Gochang, South Korea. The infected stems turned dark brown, then became gray and died. Of five stem isolates taken, all were identified as *C. gloeosporioides* based on morphological and cultural characteristics.

Although no cultivar names were provided, it is assumed the infected plants were northern highbush because of the climate in Gochang, South Korea, which is located at latitude 35° N. In a separate study, stem blight was observed on highbush blueberry in Japan, with the previous year's shoots turning brown and then blighted, along with the death of adjacent floral buds. In addition, small red or brown leaf spots were observed near the blighted stems. Isolates from stem tissue were identified as *Colletotrichum acutatum* based solely on morphological and cultural characteristics (Yoshida and Tsukiboshi, 2002).

In addition, in 2010 lesions on green northern highbush blueberry canes were reported in Michigan. The lesions were described as dark brown to black, circular or oval, with light brown to gray centers, and salmon-pink masses of spores. The pathogen was identified as *C. acutatum* by morphological characteristics. This report also noted that anthracnose stem lesions had been observed on northern highbush blueberry in Ontario, Canada, and Michigan in 2003 and 2004 (Schilder, 2010). Finally, in 2013, stem lesions and leaf spots on highbush blueberry caused by anthracnose were reported in Liaoning, China. It is assumed the symptomatic plants were northern highbush, consistent with the authors' use of the term “highbush blueberry” (not “southern highbush blueberry”) and the location of Liaoning near latitude lat. 42°N, the same latitude as Michigan. Symptoms were described as yellow to red irregularly shaped lesions on stems and leaves, which expanded and turned dark brown, surrounded by a red halo. Isolates were identified as *C. gloeosporioides* based on morphological and cultural characteristics, which was later confirmed through molecular methods (Xu et al., 2013).

Until recently, there have been no peer-reviewed reports of anthracnose stem lesions on SHB. ‘Flicker’ is an SHB cultivar frequently selected by growers in Central and South Florida. Characteristics favoring production of ‘Flicker’ in this region are that it has very low chilling requirements, can be grown in an evergreen management system, and tends to ripen early. In 2014, several blueberry farms in Central Florida experienced issues with anthracnose stem lesions and twig dieback on ‘Flicker’, and to a lesser extent on ‘Scintilla’, a progeny of ‘Flicker’ (Harmon, 2014a). Using molecular methods, isolates taken from infected plants on four farms in this area were confirmed to be *C. gloeosporioides* (Velez-Climent and Harmon, 2016). Further compounding the disease issue, isolates of this pathogen collected on these farms have been found to be resistant to fungicides in the quinone outside inhibitor class (also called strobilurins), including azoxystrobin and pyraclostrobin (Harmon, 2014b). This outbreak of disease has resulted in a reduction in new plantings of ‘Flicker’ and ‘Scintilla’ and the removal of some existing plantings (P.F. Harmon, personal communication). Because of the early success of ‘Flicker’,

Received for publication 20 Feb. 2018. Accepted for publication 5 May 2018.

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Fig. 1. Anthracnose stem lesion on blueberry.

it has been used as a parent in the University of Florida blueberry breeding program in the past, raising concerns regarding potential susceptibility of offspring from these crosses. In addition, there is a concern about whether other commercial cultivars may be susceptible to this fungicide-resistant form of anthracnose. This could potentially lead to similar problems as those experienced with 'Flicker' and 'Scintilla', including a possible rejection by growers of further use of such cultivars and the costly removal of existing plantings.

The objective of this study was to determine the level of anthracnose susceptibility of certain commercially available SHB cultivars, so that University of Florida (UF) personnel could use any identified susceptibility to limit the use of susceptible cultivars in the UF blueberry breeding program and communicate the findings to growers for their use in making decisions on which cultivars to plant.

Materials and Methods

Plant material. Plant material used in the evaluation of anthracnose susceptibility consisted of 10 cultivars of interspecific crosses of *V. corymbosum* (SHB), all of which are commercially grown in Florida. These included 'Chickadee', 'Emerald', 'Farthing', 'Flicker', 'Jewel', 'Kestrel', 'Rebel', 'San Joaquin', 'Springhigh', and 'Star'. All plants were purchased from Fall Creek Nursery in Lowell, OR, with the exception of 'Flicker', which was purchased from AgriStarts in Apopka, FL. Both of these nurseries propagate plants from tissue culture, and they were selected to minimize any issues with latent plant infections. The plants were ≈ 10 cm tall, in 38-cell trays. All of the cultivars with the exception of 'Rebel' were developed by the University of Florida blueberry breeding program. The plants were transplanted into 15-cm Kord plastic pots (HC Companies, Middlefield, OH), filled with 100% Fafard peat (Sun Gro Horticulture, Agawam, MA), and allowed to grow for 4 months before inoculation. Plants were each placed 11.5 cm apart, within rows that were also 11.5 cm apart on greenhouse benches. The plants were watered regularly, and fertilized with a Peters Professional 20–20–20 fertilizer

(Scotts, Marysville, OH) every 2 weeks. The experiments were conducted in a temperature-controlled greenhouse without supplemental lighting at the University of Florida in Gainesville, FL. Greenhouse temperature ranged between 16 and 36 °C, measured using a HOBO temperature logger (HOBO U23-002; Onset Computer Corporation, Bourne, MA).

Experimental design. The experiment was arranged in a randomized complete block design with 10 replications, one plant per cultivar per replication. The experiment was conducted twice, both during May 2016. The treatments included 10 different cultivars of *V. corymbosum*, all of which were inoculated with a single isolate of *C. gloeosporioides*. To increase the number of replication that would be inoculated with the pathogen, 50 'Flicker' plants (known to be susceptible to anthracnose) were used as susceptible standards (sprayed with distilled water instead of the pathogen), instead of using an equal number of control replications for each cultivar. The results of spraying the susceptible 'Flicker' plants with distilled water were then compared with the pathogen-inoculated 'Flicker' plants to determine whether additional control replications (i.e., cultivars sprayed with distilled water instead of being inoculated with the pathogen) were required.

Source of isolate and inoculum preparation. The pathogen used for inoculation was a single-conidium isolate of *C. gloeosporioides* (15–646), originally isolated in 2015 from naturally infected stems of 'Flicker' on a commercial blueberry farm in Central Florida (P.F. Harmon, personal communication). The isolate was incubated on autoclaved potato dextrose agar (PDA) under continuous fluorescent lighting at 25 °C for 5 d, at which point sufficient sporulation had occurred. The petri plate containing the pathogen was flooded with distilled water, the conidia dislodged into a suspension with a sterilized, L-shaped, glass rod, and the suspension filtered through two layers of cheesecloth to remove dislodged mycelia. The conidial concentration was determined using a hemocytometer (Bright-Line Hemacytometer; Hausser Scientific, Horsham, PA) and then diluted with distilled water to obtain a conidial suspension with 1×10^5 conidia mL⁻¹. The control plants were sprayed with distilled water.

Inoculation and measurement of lesions. Inoculation was performed on 12 May and 26 May 2016. The conidial suspension was sprayed until runoff onto each plant using a Crown Spra-Tool aerosol spray gun (Aervoe, Gardnerville, NV), coating all stem surfaces and upper surfaces of leaves. Each plant was immediately sealed inside a 30.5 × 61-cm 4-mil polyethylene bag (Uline, Pleasant Prairie, WI) along with a moistened Kimwipe (Kimberly-Clark, Irving, TX) for 16 h to maintain a moist environment conducive for infection. After removal of the polyethylene bags, the plants were hand-watered daily.

Each plant was examined daily for disease symptoms. Two criteria were used to measure the disease phenotype (i.e., necrotic stem

lesions). First, the number of lesions on each infected plant was recorded to evaluate disease incidence. Second, one representative lesion per infected plant was selected, marked, and measured with a digital caliper daily for 7 d following first observation of disease symptoms, to evaluate disease severity using AUDPC.

The trapezoidal method (Madden et al., 2007) was used for the AUDPC calculations as follows: $AUDPC = \sum_{i=1}^{n-1} [(y_i + y_{i+1})/2](t_{i+1} - t_i)$, where n = number of assessments, y = disease severity score for each plant at assessment (length of anthracnose lesion in this study), and t = time at each assessment.

After the observation period, infection by *C. gloeosporioides* was confirmed through isolations from necrotic lesion boundaries. The isolated tissue was surface disinfested with a 10% bleach solution for 1 min, followed by three water rinses of 1 min each. The isolated tissue was then placed on PDA and incubated under continuous fluorescent lighting at 25 °C until conidia covered most of the plate. Confirmation was made by comparing the morphological characteristics of the isolate with those of the original isolate.

Statistical analysis. The response variables (mean lesion number and AUDPC) were analyzed using a one-way analysis of variance (ANOVA) test to assess the effect of treatment (cultivar), using the following linear model:

$$Y = \mu + \text{block} + \text{cultivar} + e,$$

where Y is the response variable; μ is the overall mean; block and cultivar are fixed effects; and e is the random residual effect $\approx N(0, \sigma^2)$.

Tukey's honestly significant difference test was applied when the ANOVA test showed significant difference among treatments, to separate treatment means at $P = 0.05$. All analyses were conducted using the R statistical software (R Core Team, 2016).

Results and Discussion

Anthracnose stem lesions were observed on young green stems of certain cultivars in both experiments; they were primarily sub-circular, sunken, and they enlarged over time. Where multiple lesions occurred in close proximity, they coalesced and caused die-back of the stem tip, although this occurrence was rare. Orange sporulation was observed erupting from the necrotic area on some lesions.

In the first experiment, the mean disease incidence on 'Flicker' was significantly higher than for all tested cultivars except for Emerald, Farthing, and Kestrel. 'Flicker' had a mean of 0.6 lesions per plant, whereas 'Farthing' and 'Kestrel' had a mean of 0.2 lesions, and 'Emerald' had a mean of 0.1 lesions as shown in Fig. 2. In the second experiment, 'Flicker' had a significantly higher mean number of lesions per plant

(1.5) than all other tested cultivars (0, 0.1, or 0.2), as shown in Fig. 3.

In the first experiment, disease severity as measured by the mean AUDPC value for Flicker lesions was significantly higher than for all tested cultivars other than Emerald and Kestrel, as shown in Fig. 4. In the second experiment, the mean AUDPC value for Flicker was significantly higher than all other tested cultivars, as shown in Fig. 5.

No disease was observed on any of the noninoculated 'Flicker' plants in either experiment. Tissue taken from each symptomatic plant confirmed that in all cases the stem lesions were attributable to *C. gloeosporioides*, because the morphological characteristics

of the isolate from the infected plants were similar to those of the original isolate.

The lesions observed in these experiments were consistent with descriptions in published reports on anthracnose. They were subcircular in shape (Jeffries et al., 1990), sunken (Freeman et al., 1998), exhibited orange sporulation (Freeman et al., 1998), coalesced into larger necrotic areas (Jeffries et al., 1990), and resulted in stem dieback (Kim et al., 2009).

The results of this study regarding cultivar-specific susceptibility were consistent with reports from commercial blueberry growers in Central Florida, who reported anthracnose stem lesions on 'Flicker', and

to a lesser extent 'Scintilla', which is a progeny of 'Flicker' (P.F. Harmon, personal communication). In the second experiment in this study, where there was no wounding (discussed in the following paragraphs), 'Flicker' showed significantly greater disease incidence and severity following inoculation with a *C. gloeosporioides* isolate. It should be noted that we were unable to obtain 'Scintilla' plants in time for inclusion in this experiment; however, it will be included in future screening studies.

The anthracnose stem lesions in these experiments were similar in some respects to reports of stem lesions on northern high-bush blueberries in Asia and the United States. In a case of anthracnose observed on blueberry in northeastern China, the lesions were described as yellow to reddish, irregularly shaped, becoming dark brown in the center and expanding (Xu et al., 2013). A report from Japan described anthracnose lesions on blueberry stems as the shoot tips turning brown, then blighted within 20 cm of the tips. Most of these lesions remained constant in size (Yoshida and Tsukiboshi, 2002). Also, Kim et al. (2009) reported anthracnose on blueberry stems in South Korea that turned brown to dark brown, became gray, followed by stem death. Finally, a report from Michigan indicated that anthracnose lesions on green blueberry canes were dark brown to black, circular or oval, with light brown to gray centers, and salmon-pink spore masses (Schilder, 2010). The stem lesions observed in our research were dark brown to black, subcircular to oval, with orange- or salmon-colored conidia erupting from some of the lesions, and stem blight at the tips, which are all similar characteristics to some of the published reports. However, there were differences with some of the reported characteristics, including color of the lesion (yellow to reddish according to Xu et al. (2013), light brown to gray centers per Schilder (2010), stems turning gray per Kim et al. (2009), and lesions remaining constant in size per Yoshida and Tsukiboshi (2002)). The cause of these morphological differences is unknown, but it may have been due to cultivar characteristics (northern high-bush vs. SHB), pathogen species differences (*C. gloeosporioides* vs. *C. acutatum*), or environmental conditions.

As discussed previously, there were some differences between the two experiments in this study in the development of lesions on certain cultivars. In the first experiment, the mean number of lesions and AUDPC on 'Flicker' was not significantly different from certain other cultivars, but in the second experiment the results for 'Flicker' were significantly different from all other cultivars. In addition, 'Flicker' had a higher mean number of lesions in the second experiment (1.5 per plant) than in the first experiment (0.6 per plant).

In the first experiment, some of the young stems had heat damage due to high temperatures in the greenhouse, whereas the plants were covered with polyethylene bags, which may have affected the results. The greenhouse temperature during inoculation was $\approx 29^{\circ}\text{C}$

Fig. 2. Mean number of anthracnose lesions per plant, with 10 replications per cultivar—Experiment 1. Columns with the same letter are not significantly different according to Tukey's honestly significant difference test ($\alpha = 0.05$).

Fig. 3. Mean number of anthracnose lesions per plant, with 10 replications per cultivar—Experiment 2. Columns with the same letter are not significantly different according to Tukey's honestly significant difference test ($\alpha = 0.05$).

Fig. 4. Mean area under the disease progress curve (AUDPC) per cultivar—Experiment 1. Columns with the same letter are not significantly different according to Tukey's honestly significant difference test ($\alpha = 0.05$).

Fig. 5. Mean area under the disease progress curve (AUDPC) per cultivar—Experiment 2. Columns with the same letter are not significantly different according to Tukey's honestly significant difference test ($\alpha = 0.05$).

(and up to 36 °C thereafter) and would have been higher in close proximity to the plants while sealed inside the bags. Inoculation and covering of the plants with polyethylene bags was performed later in the afternoon for the second experiment, minimizing the amount of time the plants were exposed to higher temperatures while covered (temperature during inoculation was between 22 and 25 °C), and no heat damage was observed on removing the bags.

It is possible that the heat-inflicted wounding allowed *Colletotrichum* to directly infect senescent tissue of some of the plants, or opportunistically as a saprophyte, where infection otherwise may not have occurred. In particular, 'Emerald' and 'Kestrel', which

both had heat damage on some young tips, developed stem lesions in the first experiment, whereas none of the 10 replications of these two cultivars developed lesions in the second experiment where there was no wounding. There was no heat damage on any of the 'Flicker' plants. In one study where red-fleshed dragon fruit were inoculated with *C. truncatum*, they observed no anthracnose symptoms on nonwounded stems, whereas wounded stems were symptomatic. Vijaya suggested that wounds may increase a plant's susceptibility to infection through direct introduction of the pathogen into plant tissues, whereas on nonwounded stems, the cuticle may protect the plant against infection (Vijaya et al., 2015). In addition, in two reports of

anthracnose stem lesions on northern high-bush blueberry, inoculations were performed on wounded and nonwounded tissue (stems and leaves). Although the wounded tissue became infected, the nonwounded tissue showed symptoms either weakly or not at all (Kim et al., 2009; Yoshida and Tsukiboshi, 2002).

It is also possible that the high temperatures had an inhibiting effect on growth of the *Colletotrichum* isolate, resulting in fewer mean lesions per plant on 'Flicker' in the first experiment. Appressoria formation (an infection mechanism used by *Colletotrichum*) and conidial germination are affected by temperature (Dodd et al., 1991; King et al., 1997). Several studies have found that the optimal temperature for growth, conidia formation, and appressoria formation in *C. gloeosporioides* is between 20 and 28 °C, with a decrease at or above 30 °C (Dodd et al., 1991; Hartung et al., 1981; King et al., 1997; Lee, 1993). Temperatures in excess of 30 °C in the first experiment, especially during the immediate postinoculation period, may have created an environment that was not favorable for growth of the pathogen, resulting in lower rates of infection on 'Flicker'. Due in part to the lower infection rate on 'Flicker' in the first experiment, the mean number of lesions was not significantly greater than the other cultivars. In the second experiment, the disease incidence on cultivars other than Flicker was similar to the first experiment, but disease incidence on 'Flicker' was greater and less variable, resulting in a statistically significant difference as compared with other cultivars. The progress of disease severity on 'Flicker' also was significantly greater in the second experiment than on other cultivars, suggesting that susceptibility is not solely tied to success of infection but also processes related to postinfection pathogenesis. Additional research should focus on further optimizing postinoculation environmental conditions to reduce the error associated with measured disease response variables, thereby allowing a more precise estimation of differences in host susceptibility between host genotypes.

In conclusion, we found that 'Flicker' is more susceptible to a stem lesion form of anthracnose than other tested cultivars, which is consistent with field observations. Growers should take this susceptibility into account in considering which cultivars to plant, and any progeny of 'Flicker' should be screened using the protocol used in these experiments to limit the release of future cultivars with similar levels of susceptibility.

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