Reaction of Different *Vaccinium* Species to the Blueberry Leaf Rust Pathogen *Thekopsora minima*

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Abstract. Blueberry leaf rust caused by *Thekopsora minima* is a serious threat to blueberry production. To investigate the host range and characterize new sources of resistance, 15 southern highbush accessions (*Vaccinium corymbosum*), two interspecific hybrids (*V. elliotii* × *V. pallidum* and *V. corymbosum* × *V. pallidum*), and accessions from five diploid *Vaccinium* species were inoculated with an isolate of *T. minima*. Of 15, only two southern highbush accessions displayed resistance, whereas both accessions of *V. arboreum* displayed immunity against *T. minima*. Accessions of *V. darrowii* exhibited necrosis but with limited sporulation, indicating a high level of resistance. Sporulating lesions and brown spots were observed in accessions of *V. elliotii* and *V. tenellum*. Brown lesions, large pustules, and abundant sporulation were observed on *V. pallidum* accessions and their interspecific hybrids. As the lesions expanded, defoliation was observed in *V. pallidum* accessions. When tested against rabbiteye (*V. virgatum*) and southern highbush blueberries, urediniospores of *T. minima* from overwintering leaves of *V. pallidum* were found to be virulent, suggesting that *T. minima* overwinters on *V. pallidum*. Based on symptoms and scanning electron microscopy (SEM) of urediniospores, we hypothesize that *V. elliotii*, *V. tenellum*, *V. pallidum*, and *V. corymbosum* exhibit no host specificity to *T. minima*.

The United States has more than 75,000 acres of cultivated blueberries. One-third of this production is in the Southeastern region, which is on track to be a major hub of U.S. production within the next few years. Two types of blueberries, rabbiteye (*V. virgatum* Aiton. $2n = 6x = 72$) and southern highbush (species complex between *V. corymbosum* L. $2n = 4x = 48$ and *V. darrowii* Camp $2n = 2x = 24$), are grown in the region. Leaf rust caused by the fungus *Thekopsora minima* P. Syd. & Syd, previously known as *Pucciniastrum vaccinii* (Pfister et al., 2004; Sato et al., 1993), infects blueberry leaves and causes defoliation throughout the season. This may reduce plant vigor and lead to poor fruit production. The incidence of the disease has been increasing in the United States, where the pathogen has been reported in several states, including Delaware, New York, Michigan, Oregon, Hawaii, California, and

Georgia (Keith et al., 2008; Sato et al., 1993; Shands et al., 2018; Wiseman et al., 2016). In addition, leaf rust has been reported in many countries, including South Africa, Mexico, Spain, Argentina, Australia, and China (Barrau et al., 2002; Dal Bello and Perello, 1998; McTaggart et al., 2013; Mostert et al., 2010; Rebollar-Alviter et al., 2011; Zheng et al., 2017). *T. minima* is a heteroecious fungus requiring both primary and alternate host plants to complete its life cycle (Hiratsuka 1965). In the northern United States, the disease cycle begins in early summer when the windblown aeciospores spread from hemlocks (*Tsuga* spp.), an alternate host, to infect young blueberry leaves. In the southeastern United States, the urediniospores of *T. minima* are believed to survive the winter in a broad range of evergreen plant species, including native *Vaccinium* species. Since the alternate host is not present in the southeastern states, further investigation is needed to identify the inoculum source in this area. Several southern highbush cultivars retain mature leaves through the winter season to support developing berries in spring (Lyrene, 2005). However, most of the southern highbush cultivars flower in late winter before the plant produces a new leaf canopy to support developing berries (Lyrene, 2005). In previous field scouting in 2017, we detected leaf rust symptoms on overwintering leaves of the Blue Ridge blueberry (*V. pallidum*), which is native to the southeastern parts of the United States (Ballington, 2001).

All the monitored *V. pallidum* accessions were highly susceptible (E. Babiker, unpublished data). In contrast, leaf rust symptoms on cultivated blueberries was detected in the first 2 weeks of May 2017 and increases in leaf rust severity were associated with warm and humid weather (E. Babiker, unpublished data). This suggest that the urediniospores of *T. minima* spread from infected leaves of *V. pallidum* to the newly emerging leaves of southern highbush cultivars.

Development of disease-resistant cultivars relies on the characterization and incorporation of genes for resistance. The native diploid *Vaccinium* species are important sources of adaptive traits. Southern highbush blueberry cultivars possess genes introduced from several *Vaccinium* species, including *V. darrowii* Camp, *V. angustifolium* Ait., *V. virgatum* Ait., *V. elliotii* Chapm., *V. tenellum* Ait., *V. pallidum* Ait., *V. myrsinites* Lam., and *V. stamineum* Lam. (Brevis et al., 2008; Ehlenfeldt et al., 1995; Hancock et al., 1995; Yousef et al., 2014). Relatively little is known about the reaction of commercial blueberry cultivars and native diploid *Vaccinium* species to *T. minima*. To address this concern, this study was conducted to 1) determine whether *T. minima* collected from overwintering leaves of *V. pallidum* is pathogenic on rabbiteye and southern highbush blueberries; and 2) investigate the reactions of *V. darrowii*, *V. elliotii*, *V. tenellum*, *V. pallidum*, *V. arboreum*, and southern highbush accessions to an isolate of *T. minima* collected from southern highbush blueberry. Information about host resistance will help us to identify genes for resistance from different *Vaccinium* species. Genes for resistance from the native diploid *Vaccinium* species could be used to develop disease-resistant cultivars.

Materials and Methods

Light and SEM. To examine the morphological characteristics, size, and shape of urediniospores, small leaf segments bearing urediniospores of *T. minima* were floated on glass slides, covered with coverslips, pressed gently, and examined with a bright-field light microscope at ×40 (Olympus, Center Valley, PA). To verify the presence of urediniospores and investigate the spore surface morphology, leaves of *V. elliotii*, *V. tenellum*, *V. pallidum*, and *V. corymbosum* were collected from naturally infected plants at Poplarville, MS, and placed in a fixative solution consisting of 2.5% glutaraldehyde and 2% paraformaldehyde buffer. The tissues were then treated with 1% osmium tetroxide, dehydrated in graded series of ethanol before critical point drying, and examined using FEI Quanta 200F SEM (FE-SEM; FEI Company, Hillsboro, OR).

Pathogenicity of *T. minima* isolate from *V. pallidum* on cultivated blueberries. Urediniospores of *T. minima* were collected from pustules on the lower leaf surface of *V. pallidum* accession B0339 using a vacuum pump (Gamut, Chicago, IL). The spores were

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collected into a gelatin capsule, diluted in a light mineral oil (Soltrol 170; Chevron Phillips, The Woodlands, TX) to a concentration of 3×10^5 spore/mL, and sprayed homogeneously with a portable air-pump sprayer onto fully expanded leaves of two rabbiteye accessions ('Tifblue' and MS 1408) and two southern highbush accessions ['Suzibblue' (NeSmith, 2010) and MS 1425]. The experiment was conducted in 2017 and 2018 and arranged in a complete randomized block design with two replicates and four plants per accession. After inoculation, the leaves were allowed to dry for 60 min and incubated for 48 h in a growth chamber maintained at 70% relative humidity at 20 °C under 8 h of fluorescent lighting. An ultrasonic humidifier was used to maintain a high humidity level in the growth chamber. After incubation, plants were moved to a growth chamber programmed for a 16-h photoperiod at 22 °C/18 °C day/night temperature. The control plants were sprayed with the light mineral oil and incubated in a different growth chamber maintained at the same conditions. Four weeks after inoculation, four to six leaves were inspected and the disease reactions were rated as immune (0), where necrotic flecks and no sporulation were detected on the leaf surface; resistant (1), where necrosis, chlorosis, and limited sporulation were observed; moderate resistant/moderate susceptible (2), where necrosis, chlorosis, sporulation, and brown spots were observed in less than 50% of the leaf surface; susceptible (3), where brown spots and sporulating lesions were detected in more than 50% and less than 75% of the leaf surface; and highly susceptible (4), where brown spots and abundant sporulation cover more than 75% of the leaf surface and defoliation were observed.

Inoculation of different *Vaccinium* species with *T. minima*. To derive an isolate, urediniospores of *T. minima* were collected from a single pustule on the leaf surface of the southern highbush blueberry cultivar Suzibblue using a vacuum pump. The spores were increased on the southern highbush accession MS 1177 following the inoculation protocol described previously. The freshly collected urediniospores were used to inoculate two accessions each of *V. darrowii* ($2n = 2x = 24$), *V. elliotii* ($2n = 2x = 24$), *V. tenellum* ($2n = 2x = 24$), *V. arboreum* ($2n = 2x = 24$), and *V. pallidum* ($2n = 2x = 24$). In addition, 15 southern highbush accessions ($2n = 4x = 48$) from different blueberry breeding programs and two interspecific hybrids, (*V. corymbosum* cv. Rubel $\times$ *V. pallidum* accession B0100) and (*V. elliotii* accession B0230 $\times$ *V. pallidum* accession B0100), were included in the test. The same inoculation protocol was followed as described previously. After inoculation and incubation, plants were placed in two growth chambers programmed for a 16-h photoperiod at 22 °C/18 °C day/night temperature. The 2017 and 2018 experiments were arranged in a complete randomized block design with two replicates and four plants

per accession. Leaf rust severity was assessed 28 d after inoculation using the rating scale described previously.

Statistical analysis. Analysis of variance was calculated using proc GLM in SAS 9.4 (SAS Institute, Cary, NC). The two experiments were conducted twice and means of disease rating displayed by different *Vaccinium* species were compared using Fisher's protected least significant difference at $P \leq 0.05$.

Results

Light and SEM. Light microscopy showed that the urediniospores were obovate with an echinulate wall and measured $17.1\text{--}27.2 \times 12.3\text{--}17.3 \mu\text{m}$ (Fig. 1). No teliospores were detected on the examined samples. FE-SEM observation showed that the lower leaf surface of *V. pallidum* was colonized by urediniospores of *T. minima* (Fig. 2). In addition, FE-SEM revealed that the morphology of the urediniospores produced from pustules on *V. elliotii*, *V. tenellum*, and *V. pallidum* could not be differentiated from the urediniospores from *V. corymbosum* (Figs. 3 and 4). Spore sizes and morphology were congruent with *T. minima* described previously (Keith et al., 2008; Shands et al., 2018; Wiseman et al., 2016).

Pathogenicity of *T. minima* isolate from *V. pallidum* on cultivated blueberries. A compatible infection type was observed between the *T. minima* isolate from *V. pallidum* and all the tested blueberries accessions. Compared with the noninoculated control, *T. minima* from *V. pallidum* caused leaf rust symptoms on all tested rabbiteye and southern highbush blueberries accessions ($P < 0.0001$). Disease symptoms first appeared as necrotic spots on the upper leaf surface of infected plants and as the infection progressed, tan to reddish-brown spots enlarged and covered the leaf surface. Urediniospores developed on the lower surface of older leaves as yellow–orange pustules within 10 to 14 d of infection and abundant sporulation across the lower leaf surface was observed. The disease symptoms displayed by these accessions were similar to the ones described in previous leaf rust reports (Barrau et al., 2002; Dal Bello and Perello, 1998; Keith et al., 2008; McTaggart et al., 2013; Mostert et al., 2010; Rebollar-Alviter et al., 2011; Shands et al., 2018; Wiseman et al., 2016; Zheng et al., 2017).

Inoculation of different *Vaccinium* species with *T. minima*. The reaction of different *Vaccinium* species to *T. minima* was assessed following the disease rating scale described previously. Because there was no significant effect of years for disease rating, data from the two trials (2017 and 2018) were pooled and analyzed together. Significant variation in virulence to *T. minima* was detected between *Vaccinium* species ($P < 0.0001$). Least significant difference showed that *V. pallidum* accessions exhibited the most susceptible reaction to *T. minima* followed by their interspecific hybrids, *V.*

Fig. 1. Urediniospores of *Thekopsora minima* with dense spinules collected from the lower leaf surface of *Vaccinium pallidum*. Bar represents 20 μm .

Fig. 2. Scanning electron micrograph showing ruptured leaf epidermis and urediniospores of *Thekopsora minima* inside leaf tissue of overwintering *Vaccinium pallidum*.

corymbosum, *V. tenellum*, and *V. elliotii*, respectively (Table 1). Significant differences between *V. corymbosum* accessions were observed for susceptibility to *T. minima* ($P < 0.007$). Of the 15 southern highbush accessions tested, only two accessions, MS 1718 and PI 638745, displayed resistance to the isolate of *T. minima* (Table 2). Limited sporulation surrounded by extensive yellow/green islands were observed on leaf surfaces of the tetraploid *V. corymbosum* accessions PI 638745 and MS 1718 (Fig. 5A and B), indicating a resistant disease response to *T. minima*. The southern highbush blueberry accessions 'Windsor', 'Pearl', 'Bobolink', 'Ventura', 'O'Neal', 'Suzibblue', 'Spring-high', MS 1425, MS 1177, 'Snowchaser', 'Biloxi', 'Sharpblue', and 'Star' displayed moderate-to-high levels of susceptibility to the isolate of *T. minima* (Table 2). After inoculation with *T. minima*, small-sized pustules surrounded by necrotic areas with clear sporulation and covering less than 50% infected area were observed on leaf surfaces of the *V. tenellum* and *V. elliotii* accessions (Fig. 5C to F) indicating moderate levels of susceptibility. Large pustules without chlorosis and high levels of sporulation were observed in all tested *V. pallidum* accessions (Fig. 5G and H). Four weeks after inoculation, disease severity was high on all *V. pallidum* accessions, and most of these plants were defoliated. When tested against *T.*

Fig. 3. Urediniospores of *Thekopsora minima* with dense spinules on the lower leaf surface of (A) *Vaccinium corymbosum*, (B) *Vaccinium tenellum*, (C) *Vaccinium elliotii*, and (D) *Vaccinium pallidum*.

Fig. 4. Leaf rust pustule with urediniospores of *Thekopsora minima* inside leaf tissue of (A) *Vaccinium corymbosum*, (B) *Vaccinium tenellum*, (C) *Vaccinium elliotii*, and (D) *Vaccinium pallidum*.

minima, the two interspecific hybrids (*V. corymbosum* cv. Rubel $\times$ *V. pallidum* accession B0100 and *V. elliotii* accession B0230 $\times$

V. pallidum accession B0100) showed yellow spots covering more than 75% of the upper leaf surface (Fig. 6A), medium pustules

without chlorosis, and high levels of sporulation on the lower leaf surface (Fig. 6B). These two tested interspecific hybrids displayed a higher level of susceptibility to *T. minima* compared with one of the parent species, *V. elliotii* accession B0230, which showed a moderate level of susceptibility. The two tested accessions of *V. darrowii*, accession B0002 and cv. Rosa Blush, exhibited a high level of resistance to *T. minima*, and detected pustules were small and surrounded by a necrotic area with a low rate of sporulation (Fig. 5I and J). A low rate of sporulation was common in all tested *V. darrowii* accessions. After artificial inoculation of two accessions of *V. arboreum* (accession B0059 and accession B0096) using the isolate of *T. minima* from southern highbush blueberry, no symptoms were observed except necrotic areas with no visible sporulation, indicating that *V. arboreum* is immune to *T. minima* (Fig. 5K and L).

Discussion

Based on light microscopy and SEM, we did not detect any morphological differences between *T. minima* urediniospores produced from pustules formed on leaves of *V. elliotii*, *V. tenellum*, *V. pallidum*, and *V. corymbosum*. With respect to the symptoms on tested blueberry accessions, no differences in pathogenicity or virulence were observed when tested against *T. minima* isolates from either of *V. pallidum* or *V. corymbosum*, suggesting no host specificity. Thus, *V. pallidum* could serve as the primary local source for the *T. minima* inoculum in the southeastern parts of the United States. In previous disease reports (Barrau et al., 2002; Rebollar-Alviter et al., 2011; Zheng et al., 2017), leaf rust symptoms were observed 10 to 15 d after inoculation. However, in this study, we noticed that it was difficult to discriminate between susceptible and highly susceptible accessions after 15 d and as the lesions expanded, defoliation was observed in highly susceptible accessions. Therefore, the disease reactions were rated 28 d after inoculation using a visual rating scale.

Detailed knowledge about the pathogen's host range will be useful in disease management. To address this question, an isolate of *T. minima* from *V. corymbosum* was used in a pathogenicity test against different diploid *Vaccinium* species native to the region. The pathogenicity test indicated that *T. minima* from *V. corymbosum* is virulent on *V. elliotii*, *V. tenellum*, *V. darrowii*, *V. pallidum*, *V. corymbosum*, and interspecific hybrids (*Vaccinium* spp.) on section *Cyanococcus* but no virulence was detected on *V. arboreum*, which belongs to the section *Batodendron*. In previous field scouting, natural leaf rust infections were not detected on sparkleberry (*V. arboreum*), which is native and widely abundant in the region, often growing adjacent to heavily infected *V. pallidum* and *V. elliotii* plantings (E. Babiker, unpublished data). These observations suggest that the host range of *T. minima* from *V. corymbosum* is restricted to *Vaccinium* species within the

Table 1. Ploidy level and mean disease rating displayed by different *Vaccinium* species tested in 2017 and 2018 against *Thekopsora minima*.

<i>Vaccinium</i> species	Ploidy level	Leaf rust rating ^z
<i>V. corymbosum</i>	4x	3.07 c ^y
<i>V. pallidum</i>	2x	4.00 a
<i>V. tenellum</i>	2x	2.56 d
<i>V. darrowii</i>	2x	0.75 e
<i>V. elliotii</i>	2x	2.30 d
<i>V. arboreum</i>	2x	0.00 f
<i>V. pallidum</i> interspecific hybrids	2x	3.56 b

^zLeaf rust severity was assessed using the 0 to 4 rating scale.

^yMeans followed by different letters are significantly different at 5% level of probability according to Fisher's protected least significant difference test. Means were calculated from two replicates and four plants per accession.

Table 2. Pedigree and leaf rust rating and SD displayed by different *Vaccinium corymbosum* accessions (2n = 4x = 48) tested in 2017 and 2018 against *Thekopsora minima*.

Accession	Pedigree	Leaf rust rating ^{z,y}
'Windsor'	FL83-132 × 'Sharpblue'	3.90 ± 0.35
'Pearl'	FL84-40 × 'Magnolia'	3.30 ± 0.46
'Bobolink'	FL00-28 × FL98-363	4.00
'Ventura'	FL00-60 × FL96-24	4.00
'O'Neal'	'Wolcott' × FL 64-15	3.40 ± 0.52
'Suziblue'	'Star' × TH-474	4.00
'Springhigh'	FL91-226 × 'Southmoon'	4.00
'Snowchaser'	FL95-57 × FL89-119	4.00
'Biloxi'	FL66-11 × US210	3.50 ± 0.53
'Sharpblue'	FL61-5 × FL62-4	3.40 ± 0.52
'Star'	'O'Neal' × FL80-31	3.60 ± 0.52
MS 1425	G 695 × MS 6	3.60 ± 0.52
MS 1177	MS 550 × US 851	3.50 ± 0.53
MS 1718	'Star' × TH471	1.00
PI 638745	Collected from the wild in Pennsylvania	1.30 ± 0.46

^zMeans were calculated from two replicates and four plants per accession.

^yLeaf rust severity was assessed using the 0 to 4 rating scale.

their smaller leaves and the ability of these species to drop leaves during dormancy suggest that they do not play major roles in the multiplication of *T. minima*. In contrast, the large overwintering leaves of *V. pallidum* could serve as a host for *T. minima* to survive when commercial blueberry cultivars are dormant from fall until early spring. Furthermore, large and abundant pustules produced on *V. pallidum* leaves could help *T. minima* to multiply and produce new inoculum. In the Southeastern region, hydrogen cyanamide has been used by several blueberry growers to stimulate vegetative bud-breaks and enhance leafing in early spring (Lyrene, 2005). However, no information is available concerning the impact of hydrogen cyanamide on survival and multiplication of *T. minima*. Further research is needed to address this concern.

Current management of leaf rust disease of blueberry relies on fungicide applications to maintain healthy leaves (Ingram et al., 2017; Lyrene, 2006). Genetic resistance is the most effective means of disease control, but resistance to leaf rust has not been investigated in *Vaccinium* species. To incorporate *T. minima* resistance into the southern highbush germplasm, knowledge of the genetic variation for resistance is required. Although there are no known commercial blueberry cultivars with resistance to the leaf rust pathogen, different responses to

Fig. 5. Leaf rust symptoms caused by *Thekopsora minima* on upper and lower leaf surfaces, respectively, of (A, B) *Vaccinium corymbosum* PI 638745, (C, D) *Vaccinium tenellum* accession B0759, (E, F) *Vaccinium elliotii* accession B0230, (G, H) *Vaccinium pallidum* accession B0100, (I, J) *Vaccinium darrowii* accession B0002, and (K, L) *Vaccinium arboreum* accession B0059.

section *Cyanococcus*. Further research is needed to test more *V. arboreum* accessions using different isolates of *T. minima* from *V.*

corymbosum and *V. pallidum* to confirm this finding. Although compatible infection types were observed on *V. elliotii* and *V. tenellum*,

T. minima have been observed among blueberries cultivars (Schilder and Miles, 2011). In the current study, we observed a range of

Fig. 6. Leaf rust symptoms caused by *Thekopsora minima* on upper and lower leaf surfaces, respectively, of (A, B) interspecific hybrid (*Vaccinium elliotii* accession B0230 $\times$ *Vaccinium pallidum* accession B0100) and (C, D) interspecific hybrid (*Vaccinium corymbosum* cv. Rubel $\times$ *Vaccinium pallidum* accession B0100).

responses among the southern highbush accessions after inoculation with an isolate of *T. minima*. Most of the tested accessions were rated susceptible to *T. minima*, and progeny of interspecific crosses of *V. pallidum* with *V. elliotii* and *V. corymbosum* resulted in hybrids with a high level of susceptibility to *T. minima*. Different responses to *T. minima* have been observed among different *Vaccinium* species with different ploidy levels, suggesting that there is no relationship between the ploidy level and virulence to *T. minima*. The high frequency of susceptible southern highbush accessions observed in this study could be attributed to the fact that several southern highbush cultivars have *V. pallidum* in their pedigree (Ballington et al., 1997). Because of the widespread susceptibility to leaf rust in southern highbush cultivars, more work is needed to identify new sources of resistance to *T. minima*. One of the southern highbush accession, MS 1718, displayed a high level of resistance to *T. minima*. Further research is needed to characterize this resistance and determine the inheritance of *T. minima* resistance in this accession. In addition, it is important to characterize the existing breeding selections before they are released or used in crossing. Southern highbush blueberry possesses genes introduced from several *Vaccinium* species (Brevis et al.,

2008). Two tested accessions of *V. arboreum* displayed immunity against *T. minima*. Despite the compatible infection type detected on leaves of *V. darrowii* accessions, the observed pustules were small and surrounded by a necrotic area, suggesting that *V. darrowii* could possess leaf rust resistance. The diploid *V. darrowii* has played an important role in breeding low chill southern highbush cultivars (Chavez and Lyrene, 2009; Luping et al., 1998). Resistance genes from the tested accessions of *V. arboreum* and *V. darrowii* should be useful in developing blueberry cultivars with resistance to *T. minima*. Incorporating the detected leaf rust resistance in *V. arboreum* and *V. darrowii* into cultivated blueberries could be achieved through interspecific crosses. Lyrene (2011) developed a tetraploid *V. arboreum* and used it to develop interspecific hybrids with southern highbush blueberry accessions. The search for resistance genes should be extended to include the tetraploid interspecific hybrids developed from these crosses.

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