Responses of Blueberry Genotypes to Infection by *Botryosphaeria dothidea*

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ABSTRACT

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Host tissue maturity, wound age, inoculum concentration and type, and pathogenic variability were evaluated for their effects on development of blueberry stem blight. Inoculation of succulent, partially hardened stems produced lesions that developed more rapidly and reflected differences in cultivar susceptibility more accurately than inoculation of woody stems. Fewer infections occurred with increasing age of wounds, but some wounds remained susceptible even after 4 wk. Mycelial inoculations produced disease responses similar to those produced by conidial inoculations on three blueberry cultivars. A highly significant isolate × cultivar interaction occurred in an 11-isolate × 12-cultivar matrix, indicating the presence of pathogenic variability, i.e., isolate specificity for cultivars. A weaker isolate × cultivar interaction in an 11-isolate × 6-cultivar matrix indicated, however, that the degree of adaptation of pathogen genotypes to specific blueberry cultivars is not great. Isolates of *B. dothidea* were placed in two virulence groups according to their disease reactions on three cultivars. Evaluation of blueberry breeding material for resistance to *B. dothidea* should include inoculations with representative isolates from each of the two virulence groups. Accurate assessment of cultivar susceptibility should include inoculation of succulent, partially hardened stems within 24 hr of wounding and the use of either mycelial or conidial inoculum applied at a rate of 5×10^3 conidia per wound or higher.

Stem blight of highbush and rabbiteye blueberry (*Vaccinium corymbosum* L. and *V. ashei* Reade, respectively) is caused by *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & de Not. (syn. *B. ribis* Gross. & Dug.). Symptoms include rapid wilting, browning, or reddening of leaves on individual branches and eventual death of the entire plant as the fungus reaches the base (11). Diseased stems are characterized by a pecan-brown discoloration of vascular tissues, frequently occurring only on one side of an affected stem.

Witcher and Clayton (11) indicated that infection of highbush blueberry by *B. dothidea* resulted from wound inoculations. Luttrell (5) found that *B. dothidea* could infect elm stems without wounding and that infections resulting from inoculations with conidia were more severe than inoculations with mycelium. Infection of blueberry (6) and apple (1) stems by *B. dothidea* results from both wounded and nonwounded inoculations but that, in blueberry, wound penetration is required for typical disease development.

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The disease has increased in importance in North Carolina; stem blight-infected plants have increased from 9% in 1959 (11) to 23% in 1985 (2). The increased use of highly susceptible cultivars such as Bluechip and the introduction of mechanical harvesting have contributed to the increase. The rapid death of young plants has prevented the establishment of new plantings in some areas of southeastern North Carolina. Sutton and Boyne (9) and Fulkerson (3,4) found that isolates of *B. dothidea* varied in their ability to rot apple fruit. Fulkerson (3) also found that mycelial pigmentation and morphology and rate of pycnidia production varied among isolates from apple, but no designation of biotypes or races has been proposed.

The present research was done to develop an efficient and reliable method of screening for stem blight resistance in blueberry and to determine if pathogenic variation in *B. dothidea* indicated a need for classification of isolates as races or virulence classes.

MATERIALS AND METHODS

Inoculum. Eleven isolates of *B. dothidea* were collected from various host plant species throughout North Carolina from 1970 to 1985. Isolates BD-2, 9, 10, 17, 19, 24, 38, and 42 were isolated from blueberry, BD-12 from muscadine grape, BD-16 from sweetgum, and BD-480 from apple. The pathogenicity of all isolates was tested by placing aerial mycelium into a wounded stem of a susceptible blueberry cultivar (Bluechip or Croatan) and reisolating the fungus after 14–21 days. Single-conidial isolates were maintained on potato-dextrose agar (PDA) slants at 5 C. Cultures were grown on oatmeal agar (OMA) grown under continuous fluorescent lighting ($40\ \mu E\ s^{-1}\ m^{-2}$) for 2 wk at 25 C. Conidia were obtained by adding 10 ml of deionized water to OMA cultures, scraping the surface of the plate with a razor blade, and grinding the mixture in a mortar and pestle before straining it through several layers of cheesecloth. Inoculum for mycelial inoculations consisted of aerial hyphae removed from above the surface of an approximately 2-cm^2 area of a 2-wk-old OMA culture.

Plant material and inoculations. Blueberry plants were grown from rooted cuttings in 15- or 20-cm clay pots containing sand:peat (1:1, v/v). Highbush blueberry cultivars Bluechip, Blueray, Collins, Harrison, Jersey, Murphy, Rubel, and selection NC 1074; rabbiteye cultivars Centurion, Premier, Powderblue, and Tifblue; and a selection of lowbush blueberry, *V. darrowi* Camp were used in the experiments. Plants were fertilized weekly with a solution of Peter's 20-20-20 fertilizer (W. R. Grace & Co., Fogelsville, PA) and at 3-mo intervals with Osmocote 19-6-12 (Sierra Chemical Co., Milpitas, CA). All experiments were done in the greenhouse with mean daily maximum and minimum temperatures kept at 26–30 and 15–20 C, respectively. In each experiment plants were placed in a mist chamber at 95–100% RH for 72 hr after inoculation, then transferred to a greenhouse bench and arranged in a randomized complete block design. Pilot studies indicated that increase in stem blight lesion length diminished 4–5 wk after inoculation and that treatment differences changed very little after 4 wk; therefore, lesion lengths were measured after 4 or 5 wk. Discoloration of vascular tissue generally did not extend beyond the externally visible dieback symptoms in stems inoculated 4–5 wk before disease assessment; therefore, lesion extension was determined on the basis of external symptoms. Isolations were made from stem tissue at the margin of necrosis to verify the presence of *B. dothidea* in experiments where lesion length was measured as the disease reaction criterion.

Stem age. The effect of host tissue maturity on disease development was determined by inoculating partially hardened, succulent stems, about 3 mo old, from current season's growth and woody 1-yr-old stems of 2-yr-old plants

of cultivars Harrison and Powderblue. Inoculations with *B. dothidea* isolates BD-17, BD-18, and BD-19 were made by introducing aerial hyphae from OMA cultures onto the cut surfaces of wounds made by excising the upper portion of succulent (cut surface about 3–6 mm diameter) or woody (cut surface about 6–10 mm diameter) stems. Inoculation sites were then covered with Parafilm for 72 hr.

Two stems of each age were inoculated per plant, with five plant replicates per treatment. Lengths of stem blight lesions were measured 5 wk after inoculation. The experiment was repeated once with the addition of cultivar Murphy.

Wound age. Stems of 2-yr-old plants of the highly susceptible cultivar Bluechip were wounded by excising the upper portion of succulent or woody stems 0, 1, 7, 14, and 28 days before inoculation. A composite suspension of equal numbers of conidia from isolates BD-480 and BD-16 was used to saturate quarter segments of 6.35-mm-diameter filter paper disks. One saturated disk segment was placed on each cut stem surface, and the stem was wrapped in Parafilm. The conidial suspension contained about 5×10^6 conidia per milliliter, and each disk segment absorbed about 8 μl of the suspension, thus delivering about 4×10^4 conidia to each site of inoculation.

The first experiment was done only with succulent stems. Inoculated treatments consisted of six replicate plants with six stems inoculated on each plant. Eighteen plants wounded at the time of inoculation and receiving disk segments saturated in distilled water served as controls.

The experiment was repeated using both succulent and woody stems. Six plant replicates were used for each treatment. Five wounded stems were inoculated per plant for treatments on succulent stems, and two or three wounded stems were inoculated per plant for woody stems. Percent infection was determined by isolating from each stem 4 wk after inoculation.

Inoculum concentration and type. Conidial inoculum of isolate BD-480 at four concentrations (1, 5, 10, and 50×10^3 conidia per inoculation site) was compared with mycelial inoculum on 2-yr-old plants of Bluechip, Powderblue, and Murphy. Isolate BD-10 was tested at 1, 5, and 10×10^3 conidia per inoculation site. Mycelial inoculations were as described for the stem age tests. Conidial inoculations were made by placing a droplet containing 5–15 μl of the appropriate conidial suspension on the stem surface, piercing the stem beneath the droplet two or three times with a probe, and allowing the suspension to be absorbed by the plant's vascular system. Conidial concentration and droplet size were manipulated to deliver the desired number of conidia to each site.

The experiment was repeated with the 5×10^4 conidia per inoculation and mycelial treatment for BD-480 being omitted. In both experiments, two succulent stems were inoculated on each of four replicate plants. Before analysis, data obtained from the two experiments were transformed as $\log_{10}(n + 1)$, where n = mean lesion length (mm), to stabilize variances. Transformed data were analyzed by regression on the log of inoculum concentration levels.

Pathogenic variability. Eleven isolates of *B. dothidea* were inoculated onto 12 blueberry cultivars and selections. Mycelial inoculations were done as described in the stem age studies, and lesion lengths were measured as the criterion of disease development 4 wk after inoculation. The treatments and uninoculated controls were replicated three times; two succulent stems were inoculated on each 2-yr-old plant. The experiment was repeated with 11 isolates and with six host cultivars selected on the basis of their reactions in the first experiment. Treatments and controls were replicated four times in the second experiment.

Data were transformed as square root of $(n = 0.5)$, where n = mean lesion length (mm) before analysis. A disease reaction was considered susceptible if the mean lesion length for the treatment exceeded 10 mm. This length was considered to be of practical significance for screening for resistance. Means separation tests generally agreed with this lesion length as a breakpoint between data points for susceptible and resistant cultivar groups.

RESULTS

Stem age. The results of the two experiments were similar, and data from the second experiment are presented (Table 1). Lesion length was significantly influenced by isolate ($P > F = 0.002$), cultivar ($P > F = 0.001$), stem age ($P > F = 0.0001$), and the interactions of isolate × stem age ($P > F = 0.03$) and cultivar × stem age ($P > F = 0.002$). Lesion lengths were greater for succulent stems of cultivars Harrison and Powderblue than Murphy, with no difference between cultivars for woody stems, hence the cultivar × stem interaction.

Wound age. In the first experiment, the percent isolation of *B. dothidea* from succulent stems inoculated 0, 1, 7, 14, and 28 days after wounding was 93, 86, 84, 71, and 86, respectively. The fungus was not isolated from uninoculated controls. In the second experiment (Table 2), susceptibility decreased with increasing age of wound. *B. dothidea* was isolated from one uninoculated succulent stem control and from two woody stem controls.

Inoculum concentration and type. The

Table 1. Mean lesion length (mm) resulting from conidial inoculation of succulent or woody blueberry stems with three isolates of *Botryosphaeria dothidea*[y]

Cultivar	Isolates	Succulent stems	Woody stems
Harrison	BD-17	55 ab[z]	32 a
	BD-18	54 abc	16 a
	BD-19	61 a	14 a
Powderblue	BD-17	58 ab	29 a
	BD-18	41 abc	14 a
	BD-19	58 ab	22 a
Murphy	BD-17	32 bc	30 a
	BD-18	27 c	13 a
	BD-19	42 abc	18 a
Mean		48	21

[y] Lesion lengths (mm) were measured 5 wk after inoculation of succulent, partially hardened 3- to 4-mo-old current season's stems or 1-yr-old woody stems.
[z] Means within a column followed by a common letter are not significantly different ($P = 0.05$) according to Tukey's studentized range test.

Table 2. Isolation of *Botrosphaeria dothidea* from succulent and woody Bluechip blueberry stems after inoculation of wounds of various ages

Wound age[a] (days)	Succulent stem[b]		Woody stem[b]	
	No.[c]	%	No.	%
0	25/25	100	17/17	100
1	30/30	100	15/17	88
7	16/25	64	16/26	62
14	18/24	75	8/21	38
28	17/24	71	9/16	56
Control	1/13	8	2/8	25

[a] Age (days) of wound when inoculated.
[b] Succulent stems were 3- to 4-mo-old partially hardened current-season's growth. Woody stems were 1 yr old.
[c] Number of successful isolations/number of isolations attempted, 4 wk after inoculation.

effects of inoculum concentration and type of inoculum on stem blight development were similar in both experiments. The analysis of variance of the log-transformed data produced significant F-tests for differences among isolates and among cultivars (Table 3). All F-tests for interaction terms did not indicate significance. A contrasts analysis revealed no significant difference between the conidial and mycelial type of inoculation. Regression of the log-transformed data on the $\log_{10}$ of levels of inoculum concentration revealed a linear relationship between inoculum concentration and lesion length for Bluechip and Murphy: $Y = 0.41 \log X$, $P > F = 0.008$; and $Y = -1.11 + 0.47 \log X$, $P > F = 0.003$, respectively, where $Y = \log_{10}$ (mean lesion length + 1) and X = inoculum level, but a quadratic relationship for Powderblue: $Y = -7.30 + 4.23 \log X - 0.54 (\log X)^2$ $P > F = 0.03$. Because of high variability, R values were 29, 24, and 24% for Bluechip, Murphy, and Powderblue, respectively.

Resistant reactions were obtained for cultivars Powderblue and Murphy with mycelial inoculum and all concentrations of conidia for isolate BD-480. These cultivars were resistant to isolate BD-10 when the inoculum concentration was 1×10^3 conidia per wound but were susceptible at concentrations of 5 and 10×10^3 conidia per wound. No lesions developed on stems of the uninoculated controls.

Table 3. Effects of type and concentration of *Botryosphaeria dothidea* inoculum on development of stem blight lesions on three blueberry cultivars 1 mo after inoculation

Isolate	Inoculum type and concentration (conidia per wound)	Lesion length (mm)[a]		
		Bluechip	Powderblue	Murphy
BD-10	1×10^3	21	7	2
	5×10^3	33	23	16
	1×10^4	33	21	26
BD-480	1×10^3	10	2	0
	5×10^3	16	3	3
	1×10^4	17	5	2
	5×10^4	45	6	9
	Mycelium[b]	21	6	3

[a] Analysis of variance F-tests indicated significant differences among isolates and cultivars. All interaction term F-values were nonsignificant ($P = 0.05$).
[b] Mycelial inoculum per wound consisted of aerial hyphae removed from above the surface of an approximately 2-cm^2 area of an oatmeal agar culture. A contrast analysis revealed no significant difference ($P = 0.05$) between mycelial and conidial inoculations of isolate BD-480.

Table 4. Lesion length responses of six blueberry cultivars and selections to 11 isolates of *Botryosphaeria dothidea*

Isolate	Mean lesion length (mm) of cultivar						
	Bluechip	Rubel	Powderblue	Collins	Murphy	Premier	Means
BD-42	96[y]	54	53	48	47	39	54 a[z]
BD-38	49	39	34	42	36	31	39 b
BD-17	58	24	25	22	51	31	37 b
BD-19	61	31	43	29	20	30	36 b
BD-10	28	15	10	17	13	21	17 c
BD-2	17	8	6	14	4	11	10 d
BD-16	27	8	8	6	9	2	10 d
BD-12	31	7	3	4	5	5	9 de
ND-480	16	9	6	2	1	16	8 def
BD-9	21	14	5	2	1	1	7 ef
BD-24	15	6	5	3	5	4	6 f
Means	37 a	20 b	19 bc	18 bc	17 c	16 c	

[y] Mean of eight observations.
[z] Means followed by a common letter within overall cultivar or isolate means are not significantly different according to the Waller-Duncan K-ratio t test ($P = 0.05$).

Table 5. Disease reactions of three differential blueberry cultivars to 11 isolates of *Botryosphaeria dothidea*

Isolate	Mean lesion length (mm) of cultivar		
	Bluechip	Murphy	Powderblue
Virulence class 1			
BD-42	96 S[a]	47 S	53 S
BD-38	49 S	36 S	34 S
BD-17	58 S	51 S	25 S
BD-19	61 S	20 S	43 S
BD-10	28 S	13 S	10 S
Virulence class 2			
BD-2	17 S	4 R	6 R
BD-16	27 S	9 R	8 R
BD-12	31 S	5 R	3 R
BD-480	16 S	1 R	6 R
BD-9	21 S	1 R	5 R
BD-24	15 S	5 R	5 R

[a] Mean lesion lengths <10 mm were considered resistant (R); those >10 were considered susceptible (S).

Pathogenic variability. In the first experiment, highly significant differences among isolates and cultivars ($P > F = 0.0001$) for the isolate × cultivar interaction occurred in the analysis of variance for lesion length when 12 blueberry cultivars were inoculated with 11 isolates of *B. dothidea*. The mean and range of lesion lengths (mm) of the 12 blueberry cultivars in response to 11 isolates of *B. dothidea* 1 mo after inoculation were as follows: BD-19 (45, 24–61), BD-38 (37, 15–46), BD-17 (35, 22–53), BD-42 (27, 2–57), BD-10 (20, 6–32), BD-16 (17, 7–34), BD-12 (14, 5–27), BD-24 (14, 4–40), BD-2 (11, 7–23), BD-480 (8, 2–22), and BD-9 (7, 2–14). The mean lesion length (mm) in Bluechip, Blueray, NC 1074, Jersey, Tifblue, Rubel, Collins, Murphy, Powderblue, Centurion, *V. darrowi*, and Premier in response to the 11 isolates of *B. dothidea* was 33, 29, 23, 23, 22, 22, 21, 21, 19, 16, 14, and 13, respectively.

The six cultivars selected for further testing in the second experiment to represent the range of susceptibility identified in the first experiment. Again the analysis of variance revealed highly significant isolate and cultivar differences ($P > F = 0.0001$), but the isolate × cultivar interaction reflected a weaker interaction and fewer changes in ranking of isolates (Table 4) than in the first test. On the basis of disease reactions of the differential cultivars Bluechip, Murphy, and Powderblue (Table 5), the 11 isolates may be separated into two virulence groups. Group 1 contains the isolates BD-42, 38, 17, 19, and 10 to which little resistance is expressed by the three cultivars. In group 2, Murphy and Powderblue show resistance to the other six isolates, BD-2, 480, 16, 12, 9, and 24.

DISCUSSION

The age or maturity of stems strongly affected the rate of lesion development of *B. dothidea* on blueberry. The slower rate of death of woody stems is a common phenomenon in several host-pathogen

systems. Weaver (10) determined that 1- or 2-yr-old peach branches inoculated with *B. dothidea* showed symptoms of gummosis more rapidly than 3-yr-old branches, although the degree of symptom expression became nearly equal over time. In our study, inoculation of succulent blueberry stems revealed differences among cultivars and isolates that were not apparent with woody stems. These differences correspond well to data from field surveys of the relative susceptibility of cultivars to the pathogen (2).

The general trend toward fewer infections occurring with increasing wound age probably reflects a wound healing response. These results agree with those of Schreiber (7), who found that pruning wounds on rhododendron decreased in susceptibility to *B. dothidea* over a period of 8 wk.

Cultivars responded similarly to conidial and mycelial inoculations of isolate BD-480. The responses of the three cultivars to the higher concentration conidial inoculations of isolate BD-10 were similar to the mycelial inoculation response for this isolate in the pathogenic variation experiments.

Screening for stem blight resistance in blueberry relies on field observation, but results of this study suggest that choice of host tissue, age of wound, and to a much lesser extent, inoculum type can affect the responses of blueberry stems to inoculation with *B. dothidea* and hence the evaluation of breeding material. Succulent stems that are partially hardened (about 3–4 mo after new growth occurs) will more accurately reflect the susceptibility of the cultivar than either older or very young stems. Inoculation of mechanically wounded stems within 24 hr of wounding allows a higher percentage of positive inoculations than other means of wounding or inoculating older wounds. Mycelial inoculation is equivalent to the higher concentrations of conidial inoculum and may be used successfully in assessment of susceptibility or resistance of blueberry breeding material.

Blueberry cultivars vary widely in their responses to infection by *B. dothidea*, and isolates of the pathogen show a broad range of pathogenicity. This variability markedly affects the type of disease response seen in any specific isolate and cultivar combination. Variation in pathogenicity of isolates of *B. dothidea* is at least partially specific for cultivar, as is revealed by the highly significant isolate × cultivar interaction found in the 11-isolate × 12-cultivar matrix. The weaker interaction ($P > F = 0.08$) found in the 11-isolate × 6-cultivar matrix indicates, however, that the degree of adaptation of pathogen genotypes to specific host cultivars is not great. All the isolates were capable of infecting each cultivar tested.

Variation in pathogenicity of isolates of *B. dothidea* has been reported by several researchers. Sutton and Boyne (9) found that isolates differed in their ability to rot apple fruit, although pathogenicity on a range of apple cultivars was not tested. In a susceptibility study involving 18 blueberry cultivars and six isolates of *B. dothidea*, Milholland (6) found that one isolate was less pathogenic than the others on both highbush and rabbiteye cultivars. Schreiber (7) noted that an isolate of *B. dothidea* from rhododendron produced much larger cankers on rhododendron than did isolates originating from apple or American holly. In contrast to the foregoing information, a knowledge of the wide host range of *B. dothidea* (8) might lead one to speculate that the pathogen would show little specificity for cultivars.

On the basis of the disease reactions of cultivars Bluechip, Murphy, and Powderblue, we have classified our 11 isolates into two virulence groups. A range of isolates should be used in screening blueberry breeding material for resistance to the stem blight pathogen, and recognition of these two isolate groups indicates that representative isolates from each should be included in screening programs. A much more extensive program of evaluation of isolates from different geographic locations will be required to more accurately determine the degree of pathogenic variation and to ascertain whether races of the pathogen may be defined.

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