The Histology of Hybrid Poplar Clones Inoculated with *Septoria musiva*

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ABSTRACT

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Septoria musiva causes stem cankers that severely limit production of susceptible hybrid poplars in eastern North America. A field experiment was conducted with resistant clone DN34 and susceptible clone NC11505 in order to (i) identify tissues colonized by the pathogen, (ii) describe tissue responses to *S. musiva*, and (iii) determine whether tissue responses to *S. musiva* differed between hybrid poplar clones. Branches of each clone were inoculated by removing the fourth or fifth fully expanded leaf and placing an agar plug colonized by an aggressive isolate of *S. musiva* over the wound. Seven weeks after inoculation, branches were harvested and prepared for histology. Data from nonwounded control, wounded control, and wounded and inoculated stems were collected and analyzed for effects of clone and treatment. In general, fungal colonization was more extensive in NC11505 and exophylactic and necrophylactic periderms (NPs) of clone DN34 were significantly thicker than those of NC11505, regardless of treatment. The number of NPs produced and the distance from the epidermis to the outermost layer of phellem were significantly affected by the pathogen. Inoculated stems of clone DN34 developed a single NP that formed closer to the wound surface than in wounded controls. In contrast, inoculated stems of NC11505 developed successive NPs and the first NP formed further from the wound surface than in wounded controls. These two host responses to inoculation, as well as measures of exophylactic and necrophylactic periderm thickness, may be useful as markers for the selection of poplar resistant to damage by *S. musiva*.

The development of intensively managed poplar (*Populus* spp. and hybrids) plantations in the eastern United States and Canada has been hindered by the leafspot and canker pathogen, *Mycosphaerella populorum* (anamorph = *Septoria musiva*) (3,35). Infection of branches and stems of otherwise productive but susceptible poplar species and hybrids can lead to stem defects, breakage, and tree death. In western North America, the pathogen is reportedly absent, and short-rotation intensive-culture poplar plantations can be used as sources of fiber and lumber (20).

Poplar clones vary in response to infection and colonization by *S. musiva* or exhibit a range in canker disease damage that has sometimes been attributed to the pathogen (3,23,27,29,35,39). In addition, previous studies by our group quantified variation in canker incidence and severity

(resulting from inoculation of juvenile poplar stems with *S. musiva*) and related these to longer-term canker disease damage (36,37). However, because most studies have been based on observations of gross canker morphology, detailed information on the tissues colonized by *S. musiva* and the histological responses of poplar stems to colonization is limited and contradictory. Several researchers found that colonization was limited to the phloem, cortex, and periderm (3,33,39), and others have reported successful pathogen isolation from the xylem of infected stems (22,34,35).

A field study was conducted to continue comparative analyses of resistant and susceptible poplar clones (36,37) and to identify short-term responses to *S. musiva* that may be related to long-term canker disease damage. Stem samples of resistant clone DN34 and susceptible clone NC11505 were collected 7 weeks after inoculation with *S. musiva* and compared. Data collected described attributes considered in other woody plant-canker pathogen interactions (9–11,13,15,21), including constitutive tissues reported to influence resistance (exophylactic periderm thickness); cell proliferation and expansion as a response to wounding and inoculation (stem diameter and circumference); restoration of stem integrity (necrophylactic periderm

length and quantity, and distance from the epidermis to layers of the necrophylactic periderm); and to the presence of phloem fiber bundles, which have been reported to interrupt the necrophylactic periderm created during the restoration process. The objectives of this study were to (i) clarify which tissues were colonized by the pathogen, (ii) describe tissue responses to inoculation with *S. musiva*, and (iii) determine whether tissue responses to inoculation with *S. musiva* differed between hybrid poplar clones. The null hypothesis tested was that histological responses to wounding and inoculation with *S. musiva* do not differ between resistant and susceptible clones.

MATERIALS AND METHODS

Field design and establishment. In 2001, nine dormant cuttings each of resistant standard DN34 (*Populus deltoides* × *P. nigra*) and susceptible standard NC11505 (*P. maximowiczii* × *P. trichocarpa*) were planted into each of two field plots on the campus of the University of Wisconsin-Madison. Cuttings were planted in a completely randomized design on a spacing of 0.6 by 1.2 m. Subsequent plot maintenance included both mechanical and chemical (glyphosate) management of competing vegetation.

Inoculation. Inoculum was produced from a single-conidial isolate of *S. musiva* (isolate 92-49A, DAOM 229444) as described by Weiland et al (36). One branch on each of six trees per clone per plot was inoculated by removing either the fourth or fifth fully expanded leaf and placing a plug of inoculum on the wound with the mycelium side toward the stem (23,27). The inoculum plug was held in place using Parafilm (American National Can, Menasha, WI). Two branches on each of the remaining three trees for each clone and plot were used as controls. One branch on each control tree was used as a non-wounded control by leaving the fifth fully expanded leaf intact. The second branch on each control tree was used as a wounded control by applying a sterile malt extract agar plug onto the wound where the leaf had been removed. Trees in plot 1 were wounded and inoculated on 18 July 2002, and those in plot 2 on 24 July 2002 to provide an experimental repeat.

Histology. Branches from each treatment (nonwounded control, wounded control,

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trol, and wounded and inoculated stems) and clone in each plot were collected 7 weeks after inoculation. A 1-cm-long stem segment, centered on the inoculation point, was harvested and placed into 10 ml of formalin-acetic acid-70% ethanol (FAA, 5:5:90). Samples were dehydrated through a tertiary butanol series and then embedded with Paraplast Plus (Fisher Scientific Co., Houston). The distal end of each embedded stem segment was exposed by cutting with a razor blade and samples were soaked in 1% sodium dodecyl sulfate and glycerol (9:1) for 24 h and then in distilled water to soften tissues prior to sectioning. Samples were sectioned to 15 μ m along the transverse axis on a rotary microtome and mounted on a glass microscope slide with Haupt's adhesive without phenol. Some of the sections then were dewaxed with *d*-limonene and stained with safranin and fast green as described by Sass (31). After a final dehydration through an ethanol and xylene series, coverslips were mounted with Permount (Fisher Scientific, Pittsburgh).

Hyphae in both clones were visualized from other, nonstained sections obtained from immediately below each inoculation point by using fluorescein-labeled wheat germ agglutinin (Vector Laboratories, Inc., Burlingame, CA) as described by Morrell et al. (26) and McManus et al. (24). Sections were examined using an Olympus BH2 microscope with a BH2-RFC reflected light fluorescence illuminator (Exciter filter BP490 $\pm$ 20, dichroic mirror DM500, and Olympus B Block long pass barrier filter AFC+O-515).

Data were collected from three transverse sections per branch. One section was centered on the inoculation point, with the other two sections located approximately 225 μ m above and below this point. The diameter of the stem was measured through the inoculation point to the opposite side (diameter 1). A second diameter measurement (diameter 2) was taken through the center of the stem along the axis perpendicular to diameter 1. Each lobe of the five-sided pith was numbered clockwise from 1 to 5, with 1 assigned to the lobe just internal to the inoculation point in order to standardize for cell or tissue location in the stem cross section (Fig. 1A). The length of the outer stem perimeter from lobe 2 to lobe 5 was measured and the number of fiber bundles along this length was counted. This number was standardized by dividing the number of fiber bundles by the length of perimeter measured in each stem. The thickness of the exophylactic periderm (including the epidermis) was measured at three locations: external to lobes 2 and 5, and from the region between lobes 3 and 4. The length of the first- and subsequently formed necrophylactic periderms (NPs) were measured and a ratio (periderm ratio) was calculated as the sum of the length of

all NPs divided by the length of the first-formed NP. The distance from the epidermis to the outermost layer of the phellem (including that formed at the abscission layer in nonwounded stems), distance to the innermost phellem cell layer, distance to the innermost layer of the phellogen, and thickness of the phellem were recorded from three locations within each section (adjacent to each of the three leaf traces). Phellem-layer thickness of the NP was determined by subtracting the distance to the outermost phellem layer from the distance to the innermost phellem layer. A second measurement of phellem-layer thickness was obtained using autofluorescence associated with suberin deposition (4,12). Sections for fluorescence microscopy were examined using an Olympus BH2 microscope with a BH2-RFC reflected light fluorescence illuminator (Exciter filter BP360 $\pm$ 50, dichroic mirror DM400, and Olympus U Block barrier filter L420).

Data were analyzed by two-way analyses of variance (ANOVA) for effects of plot, clone, and treatment. If plot effects were detected, subsequent analyses also were conducted using data for each plot separately. Multiple measurements within a section and each section within a stem were considered subsamples and were averaged within each stem. Because the petiole in nonwounded control stems had a large treatment effect on diameter 1 and on the distance to the phellem and phellogen layers, separate analyses were conducted using data only from wounded control and wound-inoculated stems. Analyses were performed using Minitab Statistical Software (release 14.1; Minitab Inc., State College, PA).

RESULTS

Anatomy of nonwounded control stems. The stem anatomy for each clone was similar to *P. tremuloides* (16,18) and for hybrid poplar clone NC11505 (reported as clone NE388) (11; Fig. 1A). Three leaf traces were visible in all sections and interrupted the NP formed in the region of the leaf abscission zone. A portion of the petiole was present external to the periderm at the leaf abscission zone in all nonwounded sections.

Anatomy of wounded control stems. Stem anatomy for each clone was similar to that observed for the nonwounded control stems except for the absence of a petiole and the presence of a completely formed NP (Fig. 1B). Unlike the NP observed in nonwounded control stems, NP continuity in wounded controls was not interrupted by leaf traces. Instead, the NP either formed around or encircled the vascular bundles within the stem (Fig. 1E and F). Occasionally, the NP split periclinally into two separate NPs that later merged to reform a single periderm.

Anatomy of wounded and inoculated stems. Stem anatomy of wounded and

inoculated stems of clone DN34 was similar to that described for wounded control stems (Fig. 1C). As with the wounded control stems, NP continuity was not broken by leaf traces and, in two stems, a second NP was observed splitting from the first-formed NP. Ten stems exhibited only minor necrosis (<5% of stem cross section), with the NP formed just below the wound surface. In two stems, however, necrosis was approximately 20% and extended internally from the first-formed NP to a second NP, which delimited necrotic tissue. The second NP of these two stems extended into the phloem and formed around nearby phloem fiber bundles such that periderm continuity was maintained. In contrast, all inoculated stems of clone NC11505 developed either two or three NPs that were associated with extensive necrosis (25 to 70% of stem cross section) and cell degradation (Fig. 1D). The innermost NP either occurred in the phloem along the ring of primary phloem fiber bundles or extended to the xylem. Usually, NPs in the vicinity of the phloem formed around the fiber bundles (Fig. 1G). However, in three stems, NP continuity was interrupted at least once by their presence (Fig. 1H). In seven stems, the NP was thin and irregular in at least one location where it formed around either phloem fiber bundles or leaf traces in comparison with the NP located further from these regions. In stems where necrosis extended to the wood, NP formation terminated at the vascular cambium. Xylem vessel elements and fibers in the necrotic region were occluded.

Visualization of hyphae. Hyphae were observed in four wounded and inoculated stems of clone DN34. When present, hyphae occurred only in the necrotic epidermis and outer cortex at the wound surface. The cell lumen and pits of primary phloem fibers fluoresced brightly; therefore, the colonization of phloem fibers in clone DN34 could not be established. In contrast, hyphae were observed in all necrotic stem tissues of wounded and inoculated clone NC11505. Hyphal penetration in necrotic tissues external to the vascular cambium was primarily intercellular, with some intracellular hyphae observed. Corticular collenchyma and parenchyma were extensively colonized (Fig. 1I). As with clone DN34, the cell lumen and pits of phloem fibers fluoresced brightly. Therefore, colonization of these cells could not be demonstrated. Pycnidia and conidia, consistent with the description of *S. musiva* by Sivanesan (33), were observed in the outer cortex of six stems (Fig. 1I). Hyphae were abundant in the parenchyma of leaf traces and were visible within the associated vascular bundles, in pith parenchyma directly internal from leaf traces, and in the cell lumens of xylem vessel members and fibers (Fig. 1J).

Stem diameter. Means of diameter 1 were similar for clones DN34 and

NC11505 (Table 1). Two-way ANOVA and one-way ANOVA within each treatment were similar and indicated no effect of clone ($P = 0.801$ and $P \geq 0.623$, respectively). Diameter 1 means from wounded and wound-inoculated stems were less than those of nonwounded stems due to the removal of the petiole during the wounding process. This effect of treatment was indicated by two-way ANOVA ($P = 0.033$) but not by one-way ANOVA within each clone ($P \geq 0.107$). When diameter 1 data from nonwounded stems were omitted to account for the presence of the petiole, two-way ANOVA indicated no effect of clone ($P = 0.843$), treatment ($P = 0.415$), or interaction ($P = 0.971$). In contrast, means of diameter 2 were greater in clone DN34 than in NC11505 (Table 1). Effects of clone were indicated by two-way ANOVA ($P = 0.036$) but not one-way ANOVA ($P \geq 0.149$). Means of diameter 2 were least in nonwounded stems, greater in wounded stems, and greatest in wound-inoculated stems for both DN34 and NC11505, though an effect of treatment was observed only for NC11505 ($P = 0.019$).

Perimeter. Means of stem perimeter were similar for DN34 and NC11505 (Table 2). Two-way ANOVA and one-way ANOVA within each treatment indicated no effect of clone ($P = 0.063$ and $P \geq 0.103$, respectively). Results were similar to those of diameter 2 in that means of perimeter length were least in nonwounded stems, greater in wounded stems, and greatest in wound-inoculated stems for both DN34 and NC11505, though an effect of treatment was observed only for NC11505 ($P < 0.001$).

Number of fiber bundles. Each clone averaged four phloem fiber bundles per millimeter of perimeter (Table 2). Two-way ANOVA indicated an effect of plot with all treatments in both clones having slightly greater numbers of fiber bundles in plot 2 ($P = 0.041$). However, results were similar for each plot separately (*data not shown*); therefore, pooled data and analyses are presented. Two-way ANOVA and one-way ANOVA within each treatment and clone were similar and indicated no effect of clone ($P = 0.476$ and $P \geq 0.564$, respectively) or treatment ($P = 0.536$ and $P \geq 0.605$, respectively).

Exophylactic periderm thickness. Regardless of treatment, means of exophylac-

tic periderm thickness were more than twice as great in clone DN34 as in clone NC11505 (Table 2). Two-way ANOVA and one-way ANOVA within each treatment and clone were similar and indicated an effect of clone ($P = 0.001$ and $P < 0.001$, respectively) but not treatment ($P = 0.698$ and $P \geq 0.371$, respectively).

First-formed necrophylactic periderm length. Means of first-formed necrophylactic periderm length were similar for DN34 and NC11505 (Table 3). Two-way ANOVA and one-way ANOVA within each treatment were generally similar and indicated no effect of clone ($P = 0.146$ and $P \geq 0.123$, respectively) except for the nonwounded stems ($P = 0.005$). For DN34, the mean length of the first-formed NP in the nonwounded control stems was shorter than that in both wounded control and wound-inoculated stems. In contrast, the mean length of the NP in nonwounded control and wound-inoculated stems of clone NC11505 was shorter than that in the wounded control stems. Two-way ANOVA and one-way ANOVA within each clone were similar and indicated an effect of treatment for both clones ($P = 0.005$ and $P \leq 0.014$, respectively).

Necrophylactic periderm ratio. Means of the periderm ratio were least in nonwounded and wounded control stems and

greatest in wound inoculated stems for DN34 and NC11505 (Table 3). Two-way ANOVA indicated an effect of clone ($P = 0.005$). One-way ANOVA within each treatment indicated the clone effect occurred only with wound-inoculated stems ($P < 0.001$) and not in nonwounded or wounded control stems ($P \geq 0.494$). One-way ANOVA within each clone indicated an effect of treatment only for NC11505 ($P < 0.001$).

Distance analyses. In response to inoculation, the mean distance measured from the epidermis to each of the periderm layers (the outermost and innermost phellem layer and the innermost phellogen layer) in DN34 decreased in comparison with the wounded control stems (Table 4). In contrast, the distance to the three layers was greater in inoculated stems of clone NC11505 than in wounded control stems. The distance to all three layers always was greatest in nonwounded control stems due to the presence of petiole tissue. Two-way ANOVA and one-way ANOVA within each treatment generally were similar and indicated no effect of clone ($P \geq 0.850$ and 0.136 , respectively) except for the distance to the outermost phellem layer ($P = 0.024$). The latter was due to an effect of clone observed within the inoculated stems ($P < 0.001$) and not within the control stems

Table 1. Means of stem diameters measured from hybrid poplar clones DN34 and NC11505

Clone, treatment ^x	Variables ^a					
	Diameter 1 (mm) ^y			Diameter 2 (mm) ^w		
	Mean (n) ^y	Range	SE	Mean (n) ^y	Range	SE
DN34						
NC	5.0 (6) a	3.8–6.9	0.45	2.8 (6) a	2.2–3.7	0.24
WC	4.1 (6) a	3.3–5.0	0.26	3.0 (6) a	2.6–3.3	0.10
WI	4.3 (12) a	3.4–5.0	0.14	3.3 (12) a	2.8–3.8	0.10
NC11505						
NC	4.7 (6) a	4.3–5.2	0.16	2.4 (6) a	2.1–2.8	0.14
WC	4.2 (6) a	3.7–5.0	0.23	2.7 (6) ab	2.2–3.4	0.18
WI	4.4 (12) a	3.3–5.6	0.21	3.1 (12) b	2.3–4.3	0.17
<i>P</i> value ^z						
Clone	0.801	...	...	0.036	...	...
Treatment	0.033	...	...	0.001	...	...
Ixn	0.813	...	...	0.660	...	...

^a SE = standard error.

^y Diameter of stem as measured through the inoculation point to the opposite side.

^w Diameter of stem as measured perpendicular to diameter 1.

^x NC = nonwounded control, WC = wounded control, and WI = wound inoculated.

^y Means were calculated from three sections per stem. The number in parentheses refers to the number of stems analyzed. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference.

^z Effects of clone, treatment, and interaction (Ixn) were analyzed by two-way analysis of variance.

Fig. 1. A and B, Brightfield and **C–J,** fluorescence micrographs of transverse sections through control and inoculated 2-month-old stems of hybrid poplar clones **A** and **C,** DN34 and **B, D–J,** NC11505 stained with safranin and fast green. **A,** Nonwounded control of clone DN34 showing general stem anatomy and incomplete development of necrophylactic periderm (NP). **B,** Wounded control of clone NC11505 showing complete development of NP. **C,** Inoculated stem of clone DN34 showing development of single, complete NP. **D,** Inoculated stem of clone NC11505 showing development of 3 successive NPs. **E,** Development of NP around vascular bundle (VB) in clone NC11505. **F,** NP encircles VB in clone NC11505. **G,** Development of NP around fiber bundles (FB) in clone NC11505. **H,** NP continuity interrupted by presence of fiber bundles in clone NC11505. **I,** Pycnidia (Pc), hyphae (H), and occluded xylem elements (Xo) in colonized stem of clone NC11505. **J,** Hyphal colonization of xylem fibers in clone NC11505. Inoculation occurred from the right side of the figures in **C** and **D**, and from the top in **E–J**. Blue autofluorescence of NP viewed from ultraviolet light. Filter set parameters: BP360 $\pm$ 50, DM400, L420. Green autofluorescence viewed from blue-green light. Filter set parameters: BP490 $\pm$ 20, DM500, AFC+O-515. BT = bud trace, CTX = cortex, EP = exophylactic periderm, Pe = petiole, Pi = pith, Ph = Phloem, X = xylem, and 1 to 5 = lobes in pith. Scale bars = 100 μ m.

($P \geq 0.209$). When data from nonwounded control stems were omitted from all three parameters to account for the presence of the petiole, generally an effect of clone ($P \leq 0.039$) but no interaction ($P \geq 0.254$) was indicated. However, for the distance to the outermost phellem layer, there was an effect of interaction ($P = 0.026$) but not clone ($P = 0.076$). Two-way ANOVA and one-way ANOVA within each clone from the complete data set were similar and indicated an effect of treatment ($P = 0.001$ and $P < 0.001$, respectively). However, when data from nonwounded control stems were omitted to account for the presence of the petiole, no effect of treatment was observed ($P \geq 0.742$ and 0.106 , respectively).

Phellem-layer thickness. The NP phellem layer of DN34 always was thicker than that produced by NC11505 (Table 4). Mean NP phellem-layer thickness was least in nonwounded stems, greater in wounded stems, and greatest in wound-inoculated stems for clone DN34, but not for NC11505. Two-way ANOVA indicated an effect of plot ($P < 0.001$), with all treatments for both clones exhibiting a thinner phellem layer in plot 2. However, results were similar for each plot separately (*data not shown*); therefore, pooled data and analyses are presented. Two-way ANOVA and one-way ANOVA within each treatment indicated an effect of clone ($P = 0.001$ and $P \leq 0.005$, respectively). Analyses also indicated an effect of treatment ($P = 0.009$), which was only evident for DN34 ($P = 0.014$) and not NC11505 ($P = 0.581$). Results were similar when NP phellem-layer thickness was measured using blue autofluorescence under UV light (*data not shown*).

DISCUSSION

The observation of hyphae in xylem elements provided evidence for the colonization of wood by *S. musiva* and indicated the potential for longitudinal and lateral expansion of the pathogen through xylem elements in the leaf traces and wood. Other canker pathogens have been shown to colonize the xylem of trees (1,2,11,17,24,32). Biggs et al. (11) detected hyphae of *Cytospora chrysosperma* in the xylem of hybrid poplar clone NC11505 (reported as NE388) and McManus et al. (24) found that *Cryphonectria parasitica* colonized

the xylem of infected trees. Similarly, Simard et al. (32) detected hyphae of *Gremmeniella abietina* in the tracheids of jack pine affected by Scleroderma canker.

There are some reports that susceptibility to canker pathogens could be related to the frequency of phloem fiber bundles in the host stem (11,13,17,25). Phloem fiber bundles have been observed to interrupt the continuity of NP in colonized stems (11,13,17), which could allow pathogens to access healthy tissue. Biggs et al. (11), for example, reported that phloem fibers frequently disrupted the NP of hybrid poplar

Table 3. Means of first-formed necrophylactic periderm length and the periderm ratio from hybrid poplar clones DN34 and NC11505

Clone, treatment ^x	Variables ^a					
	Necrophylactic periderm (mm) ^y			Periderm ratio ^w		
	Mean (n) ^y	Range	SE	Mean (n) ^y	Range	SE
DN34						
NC	3 (6) a	2–4	0.22	1.0 (6) a	1.0–1.0	0.00
WC	4 (6) b	4–5	0.28	1.1 (6) a	1.0–1.4	0.06
WI	4 (12) b	3–6	0.26	1.2 (12) a	1.0–2.0	0.10
NC11505						
NC	4 (6) ab	4–5	0.14	1.0 (6) a	1.0–1.0	0.00
WC	5 (6) a	4–6	0.27	1.1 (6) a	1.0–1.5	0.08
WI	4 (12) b	3–5	0.14	1.9 (12) b	1.2–2.3	0.12
<i>P</i> value ^z						
Clone	0.146	...	...	0.005	...	...
Treatment	0.005	...	...	0.001	...	...
Ixn	0.010	...	...	0.001	...	...

^a SE = standard error.

^y The length of the first-formed necrophylactic periderm was measured as observed in transverse section.

^w Periderm ratio was calculated as the sum of the length of all necrophylactic periderms divided by the length of the first-formed necrophylactic periderm.

^x NC = nonwounded control, WC = wounded control, and WI = wound inoculated.

^y Means were calculated from three sections per stem. The number in parentheses refers to the number of stems analyzed. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference.

^z Effects of clone, treatment, and interaction (Ixn) were analyzed by two-way analysis of variance.

Table 2. Means of stem perimeter, number of fiber bundles per 1 mm of perimeter, and thickness of exophylactic periderm measured from hybrid poplar clones DN34 and NC11505

Clone, treatment ^x	Variables ^t								
	Perimeter (mm) ^u			No. FB/perimeter ^v			Exophylactic periderm thickness (μm) ^w		
	Mean (n) ^y	Range	SE	Mean (n) ^y	Range	SE	Mean (n) ^y	Range	SE
DN34									
NC	5.3 (6) a	4.0–6.4	0.42	4 (6) a	3–6	0.43	82 (6) a	69–97	4.79
WC	5.7 (6) a	5.0–6.4	0.23	4 (6) a	3–5	0.19	85 (6) a	73–94	3.32
WI	6.1 (12) a	4.3–7.6	0.24	4 (12) a	3–6	0.29	85 (12) a	64–108	3.46
NC11505									
NC	4.4 (6) a	3.8–5.6	0.29	5 (6) a	4–5	0.22	37 (6) a	34–41	1.19
WC	5.0 (6) a	4.0–6.4	0.36	4 (6) a	3–5	0.24	35 (6) a	30–41	1.71
WI	6.0 (12) b	4.0–8.2	0.39	4 (12) a	3–6	0.31	39 (12) a	30–48	1.73
<i>P</i> value ^z									
Clone	0.063	...	...	0.476	...	...	0.001	...	...
Treatment	0.004	...	...	0.536	...	...	0.698	...	...
Ixn	0.398	...	...	0.964	...	...	0.789	...	...

^t SE = standard error.

^u Perimeter of stem as measured from lobe 2 to lobe 5 of the pith. Each lobe of the five-sided pith was numbered clockwise from 1 to 5, with 1 assigned to the lobe internal to the inoculation point.

^v Number of fiber bundles (FB) counted along the stem perimeter divided by the portion of the perimeter measured.

^w Means were calculated for the thickness of the exophylactic periderm (including the epidermis) from measurements at three locations within each section (external to lobes 2 and 5, and from the region between lobes 3 and 4 of the pith).

^x NC = nonwounded control, WC = wounded control, and WI = wound inoculated.

^y Means were calculated from three sections per stem. The number in parentheses refers to the number of stems analyzed. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference.

^z Effects of clone, treatment, and interaction (Ixn) were analyzed by two-way analysis of variance.

clone NC11505 (NE388) and provided entry for the mycelia of *Cytospora chrysosperma*. In the present study, NP disruption by fiber bundles was infrequent. Most of the NPs that formed in the vicinity of the bundles in either clone formed around the fibers, which is consistent with observations by Wisniewski et al. (38) of peach stems inoculated with *C. leucostoma*. The present study also found that the NP occasionally was thinner in the region near phloem fiber bundles. This is similar to observations by Bostock and Middleton (13), who noted that NP formed in response to inoculation often was thinner in the region of phloem fibers and might allow pathogens to more easily penetrate the NP. Despite the infrequent occurrence of NP disruption and thinning in the vicinity of fiber bundles in NC11505, the number and spacing of bundles were similar to those in DN34. Therefore, no evidence was found that linked this trait to resistance or susceptibility.

Although wounding and inoculation with *S. musiva* did not significantly affect the thickness of the exophylactic periderm in either clone, the presence of a thicker periderm in clone DN34 may provide more protection against wounding and subsequent infection. In poplar, the presence of a thin exophylactic periderm has been suggested to be associated with susceptibility to wounding and infection by canker fungi (21). Stem wounds commonly provide an avenue of entry for many canker and canker rot fungi, including *S. musiva* (3,9,14,15,17,25). Although the thicker exophylactic periderm of DN34 might be a passive resistance mechanism, this was bypassed by wounding in our study. Thus, differences in responses of the two clones

studied might be attributed to other mechanisms of resistance present during infection or early postinfection in DN34.

The presence of multiple NPs in wound-inoculated clone NC11505 is similar to other reports of bark-invading pathogens (11,17,21,30,38), which were able to breach the first-formed NP and grow into healthy tissue. As the integrity of each NP was broken, new NPs were established centripetally. Biggs et al. (11) reported up to four NPs in inoculated hybrid poplar after *C. chrysosperma* exploited discontinuities in each previously formed NP. Robinson and Morrison (30) reported that *Armillaria ostoyae* can repeatedly breach NPs in naturally infected Douglas-fir and western larch. Because multiple NPs are evidence of repeated host failure to reestablish stem integrity, their presence could be used as a marker to identify susceptibility.

The effect of treatment observed in the distance analyses (distance from the epidermis to the innermost and outermost phellem layers or innermost phellogen layer) suggests some specificity of NP formation to inoculation with *S. musiva*. Some have considered phellogen restoration to be nonspecific (12,28). Mullick (28) reported that, regardless of the causal agent (i.e., mechanical wounding, insect feeding, or pathogen ingress), the restorative process was initiated whenever the extant phellogen was disrupted, and also suggested that pathogens might influence the process. More recent studies have found evidence that pathogens affect NP formation (9–11). Similar to the results of the present study, inoculation has been shown to affect the location of the NP and associated tissues in trees (9,10). Biggs

and Alm (10) observed that the distance to the ligno-suberized layer was affected in peach by inoculation with *Leucostoma cincta*. In control stems, the distance to the ligno-suberized layer (47 μ m) was almost half of that observed in inoculated stems (95 μ m).

The presence of a thick NP, as exhibited by clone DN34, has been associated with greater resistance to some pathogens in trees (5,6,30). However, Biggs (7) found that an NP was not critical in determining either resistance or susceptibility to *Leucostoma persoonii* among several genotypes of peach. If NP thickness is associated with resistance in poplar, it may be that this characteristic, like other resistance mechanisms and structures, is but one factor in the host defense repertoire and that the presence of a thick periderm alone would not confer absolute resistance.

Restoration of the phellogen after damage is critical for normal tree growth and development (8,28). Phellogen presence and activity allow the stem to accommodate secondary growth (19,28), and the resultant periderm serves as a protective tissue against the external abiotic and biotic environment (8,9,19,28). Furthermore, the phellogen is often the first living tissue affected by pathogen penetration through bark tissues (28). Mullick (28) asserted that the process of NP formation is initiated whenever the phellogen is rendered nonfunctional. Therefore, the formation of the NP in response to wounding, infection, or other forms of damage serves to restore the integrity of the stem and to reestablish the lateral meristem responsible for accommodating secondary growth (9,28). The host responses associated with reestablishment of stem integrity and success-

Table 4. Means of distance from the epidermis to three layers in the necrophylactic periderm (outermost and innermost phellem layer, innermost phellogen layer) and the thickness of the associated phellem layer

Clone, treatment ^x	Variables ^a											
	Distance to											
	Outermost phellem layer (μ m) ^y			Innermost phellem layer (μ m)			Innermost phellogen layer (μ m)			Phellem thickness (μ m) ^w		
	Mean (n) ^y	Range	SE	Mean (n) ^y	Range	SE	Mean (n) ^y	Range	SE	Mean (n) ^y	Range	SE
DN34												
NC	588 (6) a	487–831	52.1	676 (6) a	577–903	48.5	679 (6) a	586–910	50.4	86 (6) a	71–97	4.3
WC	109 (6) b	58–217	24.3	219 (6) b	158–340	26.2	227 (6) b	166–350	26.4	112 (6) ab	90–136	8.6
WI	73 (12) b	27–162	9.8	205 (12) b	120–270	12.8	212 (12) b	122–278	13.3	130 (12) b	90–201	9.7
NC11505												
NC	674 (6) a	596–827	36.6	735 (6) a	667–873	32.3	743 (6) a	676–878	31.6	62 (6) a	45–80	5.1
WC	102 (6) b	59–166	16.2	164 (6) b	96–225	21.2	172 (6) b	104–237	21.4	70 (6) a	52–91	6.5
WI	130 (12) b	94–182	8.5	189 (12) b	132–243	10.3	193 (12) b	132–248	10.9	67 (12) a	45–90	3.6
P value ^z												
Clone	0.024	...	...	0.850	...	...	0.866	...	...	0.001	...	...
Treatment	0.001	...	...	0.001	...	...	0.001	...	...	0.009	...	...
Ixn	0.194	...	...	0.091	...	...	0.080	...	...	0.041	...	...

^a SE = standard error.

^y Means for distance variables were calculated from measurements at three locations within each section (adjacent to each of the three leaf traces).

^w Means were calculated for the phellem thickness in the necrophylactic periderm from measurements at three locations within each section (adjacent to each of the three leaf traces).

^x NC = nonwounded control, WC = wounded control, and WI = wound inoculated.

^y Means were calculated from three sections per stem. The number in parentheses refers to the number of stems analyzed. Means followed by the same letter are not significantly different ($P \leq 0.05$) according to Fisher's least significant difference.

^z Effects of clone, treatment, and interaction (Ixn) were analyzed by two-way analysis of variance.

ful pathogen containment (periderm ratio, phellogen-associated distance measurements, and NP phellem thickness) were visible microscopically 7 weeks following inoculation. Clonal differences in exophylactic and necrophylactic periderm thickness were observed and may be related to resistance. Additionally, both periderm ratio and the distance from the epidermis to the outermost phellem layer were significantly different between the two clones following inoculation. Further selection of already productive poplar species and hybrids based on these and other short-term responses to inoculation (36,37) should assist in identifying clones least likely to be severely damaged by *S. musiva*.

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