# Multiple *Ceratocystis smalleyi* Infections Associated with Reduced Stem Water Transport in Bitternut Hickory

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## ABSTRACT

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Hundreds of cankers caused by *Ceratocystis smalleyi* are associated with hickory bark beetle-attacked bitternut hickory exhibiting rapid crown decline in the north-central and northeastern United States. Discolored sapwood colonized by the fungus commonly underlies the cankers. Field studies were conducted to test the hypothesis that *C. smalleyi* infections cause vascular system dysfunction in infected trees. Fifty *C. smalleyi* inoculations made at 1.8 to 3.8 m in height on stems of healthy bitternut hickory trees (13 to 28 cm in diameter at 1.4 m in height) resulted in extensive canker formation and sapwood discoloration 12 to 14 months after treatment compared with water-inoculated and noninoculated controls. Sap flow velocity (midday) was significantly lower in the infected trees compared with that in the controls. Sap flow velocity also was inversely correlated with the proportion of bark area with cankered tissues and with tylose abundance in the youngest two growth rings. Tylose formation in current-year vessels associated with *C. smalleyi* infections is likely responsible for much of the water transport disruption. It is hypothesized that multiple stem infections of *C. smalleyi* and the resulting xylem dysfunction contribute to crown wilt development in bitternut hickory exhibiting rapid crown decline.

Hickory decline, particularly decline in bitternut hickory (*Carya cordiformis*) and, to a lesser extent, in shagbark hickory (*C. ovata*), has been reported for Iowa, Maryland, Missouri, New York, Pennsylvania, West Virginia, and Wisconsin (34). Based on a 2007 to 2008 field survey of declining stands conducted in six states by J. Juzwik, three major types of symptomology were observed for smooth-bark hickory species (primarily bitternut hickory) exhibiting crown dieback or decline: (i) rapid crown decline characterized by thinning crowns with small, chlorotic leaves or foliage wilt and tree death within 2 to 3 years; (ii) top dieback with normal-sized and normal, green-colored leaves below; and (iii) slow crown decline, likely due to heavy gall formation on branches and main stems (2,12). Major biotic and abiotic factors associated with declining trees included moderate to heavy colonization of affected trees by the hickory bark beetle (*Scolytus quadrispinosis*) during or following several years of drought; much lower frequency of colonization by the hickory timber beetle (*Xyleborus celsus*); annual and diffuse stem cankers associated with *Fusarium solani* and *Ceratocystis smalleyi*, respectively; and globose galls encircling main stems and branches that were attributed to *Phomopsis* sp. infection (14). In a broad sense, these findings supported an earlier hypothesis that hickory mortality in the affected states was due to a decline complex of interacting predisposing, triggering, and contributing factors whose biotic agents are interchangeable (12,20). Alternatively, a complex of at least three diseases may be considered the cause of the widespread crown decline or dieback and mortality (14,25). Under this scenario, the most common disease found during the 2-year survey was the one characterized by rapid crown decline and subsequent tree death associated with the interaction of hickory bark beetles and *C. smalleyi*-caused cankers. The ability of *C. smalleyi* to produce stem cankers on pole-timber size (13 to 28 cm in diameter at 1.4 m of stem height [dbh]) bitternut hickory was recently published (29). Widespread mortality of hickory has historically been attributed to outbreaks of the hickory bark beetle during extended periods of drought (38). It was the discovery of a new *Ceratocystis* sp. in discolored wood and sunken bark cankers on hickory-bark-beetle-attacked trees (39) and subsequent description of the species as *C. smalleyi* (10) that led the authors to further consider the role of the pathogen in the recent occurrences of hickory mortality.

Evidence for a synergistic interaction of the hickory bark beetle and stem colonization by *C. smalleyi* associated with actively declining bitternut hickory trees was obtained by stripping the bark of three affected trees (40, 55, and 80% crown decline) and documenting frequencies and spatial occurrence of cankers and bark beetle galleries present (11,13). Numbers of bark beetle attacks ($n$ = 178, 1,448, and 991) were not correlated with crown decline rating. Full galleries (entry tunnel, egg niche, and larval tunnels) were associated with 92, 53, and 80% of the attacks, respectively. Numerous ($n$ = 26, 113, and 585) *C. smalleyi*-like cankers were found on the bark-peeled trees and correlated with the crown decline rating. The cankers were very commonly (>92%) associated with bark beetle attacks or colonization. The observed lesions in the cambium extended beyond the edges of the bark beetle damage in all cases (Fig. 1). Thus, numerous cankers as well as hundreds to thousands of bark beetle attacks are associated with bitternut hickory with active crown decline. Although synergistic interaction between the beetle and the pathogen seems to be involved, the relative importance of each of these biotic agents in causing rapid crown decline is not known. Additional studies were undertaken to consider the specific role of the pathogen in the etiology of the disease.

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figure 1 appears in color online.

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Sapwood discoloration induced by *C. smalleyi* colonization in artificially inoculated trees (13 to 28 cm dbh) extended in axial and tangential directions for ≤30 cm on the main stem from the inoculation hole but evidence of systemic spread throughout inoculated trees was not found (27). However, multiple areas of discolored sapwood often account for a large portion of the sapwood when cross sections of naturally infected stems are examined. Similar symptoms have been reported for oak (*Quercus* spp.) trees infected with *Raffalea quercivora* in Japan (17). Xylem occlusion and subsequent interruption of water transport in locally discolored sapwood of these infected oak trees has been documented. The impairment of the xylem vessels in the discolored areas results in reduced amount and rate of water transport in infected oak trees (18,21,22). Colonization of tanoak (*Lithocarpus densiflorus*) sapwood by *Phytophthora ramorum* also results in discolored sapwood and interruption of water transport in that species (30). Based on these published reports, we hypothesized that multiple stem infections by *C. smalleyi* result in xylem dysfunction and reduced water transport in bitternut hickory.

Xylem water conductance is a key physiological function with implications for tree crown condition. Hydraulic function in trees affected by diseases has been measured in different ways. Dye injection has been used to distinguish conductive versus nonconductive xylem areas and paths of water conduction in oak trees affected by *R. quercivora* (18,22). Xylem pressure potential has been measured using a pressure chamber to determine the effects of Japanese oak wilt and of pine wilt disease on tree physiology (7,21). Hydraulic conductivity has been measured to determine the extent of xylem blockage in studies on Japanese oak wilt (41) and sudden oak death (4). Sap flow monitoring systems utilizing either heat pulse velocity measurements or Granier's thermal dissipation probes have also proved useful for investigation of hydraulic function of trees (8,19,32). Reduced sap flow velocity ($J_s$) (sap flow rate per unit of conducting sapwood area) has been observed with sudden oak death in tanoak (30) and blue stain in pine and Norway spruce (15,31,40) using sap flow monitoring techniques.

**Fig. 1.** Numerous sapwood lesions visible in the cambial region and underlying Ceratocystis bark cankers on a naturally infected bitternut hickory after the bark was removed.

In 2008, field experiments were initiated utilizing Granier's thermal dissipation probes to test the hypothesis that *C. smalleyi* impairs water transport in diseased bitternut hickory. The specific objectives of our studies were to (i) evaluate differences in $J_s$ between trees with numerous cankers and canker-free trees using a sap flow monitoring system and (ii) determine the relationship between $J_s$ and (a) disease severity expressed as the proportion of the total bark area with inner bark lesions for the inoculated stem section and (b) selected xylem properties related to water transport within hickory trees. Due to the destructive nature of sampling required by the second objective, it would not be possible or appropriate to follow crown conditions of study trees beyond the period of sap flow measurement. Preliminary reports have been published (13,28).

## MATERIALS AND METHODS

**Experimental design.** An initial sap flow study using naturally declining bitternut hickory was attempted in late July 2009 in Shawano County, WI but was aborted due to data logger errors. The first experiment with artificially inoculated trees was able to proceed in September 2009 in Wabasha County, MN (44° 6′45″N; 92° 10′34″W). A mixed hardwood stand with bitternut hickory composing 35% of the stand was selected for study. Hickory mortality associated with an outbreak of the hickory bark beetle had occurred 3 to 5 years prior to initiation of this experiment. Limited equipment and travel restrictions (federal government) allowed for sap flow monitoring of only six trees. The size of the study trees was 12.6 to 27.7 cm dbh. The selected trees had healthy crowns (<15% visible dieback) and stems without visible defect or damage. The trees were intermediate to co-dominant in the canopy. The six trees were spatially distributed within a 0.5-ha area of the stand. Treatments of study trees included (i) *C. smalleyi* inoculation of three trees in July 2008, (ii) sterile water inoculation of one tree, and (iii) two noninoculated, control trees.

A second experiment with a larger number of trees was warranted based on the results of the first experiment and made possible by the fabrication of additional probes by J.-H. Park and availability of additional data loggers from J. Juzwik. The experiment was conducted in a mixed hardwood stand in Chippewa County, WI (45° 11′5″N; 91° 21′26″W). Bitternut hickory composed ≈30% of the stand following a selective thinning conducted during winter 2006–07. Hickory mortality associated with an outbreak of the hickory bark beetle had occurred 3 years before initiation of this study and had prompted the selective thinning. The size of the study trees was 16.0 to 27.3 cm dbh and trees were intermediate to co-dominant in the canopy. Each study tree had a healthy crown and a stem free of defect or damage. The 11 study trees were spatially distributed within a 1.5-ha portion of the stand. Treatments of study trees included (i) *C. smalleyi* inoculation of five trees in July 2009, (ii) sterile water inoculation of three trees, and (iii) three noninoculated, control trees.

**Fungus inoculation.** Isolates of *C. smalleyi* were obtained during 2007 from diseased trees near each of the two study sites. Two isolates were used for each site (CS0709 [GenBank accession numbers GU190738 and GU201530] and CS0729 [GenBank accession numbers GU190739 and GU201539] for Minnesota and

CS0731 and CS0734 for Wisconsin). To prepare fungal inoculum, ascospores of *C. smalleyi* were collected from extruded masses on tips of perithecia of 1- to 2-week-old cultures on 2% malt yeast extract agar and suspended in 1.0 ml of sterile distilled water. Due to the sticky nature of ascospore masses, the suspension was homogenized with a tip sonicator. The spore suspension was adjusted to a concentration of $1.0 \times 10^4$ ascospores/ml.

To mimic the numerous cankers found on naturally affected trees, multiple sites on the stem of the study tree were inoculated with *C. smalleyi*. In July 2008, 50 holes (0.6 cm in diameter) were made by aseptically drilling just into the outer sapwood between stem heights of 1.8 and 3.8 m on the main stem of four trees in Minnesota. In all, 12 to 13 holes slightly offset were drilled in each of four columns down the stem, representing the four cardinal directions. Aliquots (0.1 ml) of the fungal inoculum ($1.0 \times 10^4$ ascospores/ml) or sterile distilled water (control) were pipetted into the 50 drilled holes on an individual tree. Each inoculated hole was sealed with moist cotton and moldable epoxy putty. The noninoculated tree was selected in July 2008 but no treatment was applied. Similar treatments, except with two Wisconsin isolates, were applied to trees on the Wisconsin site in July 2009. Noninoculated trees were selected at the same time.

**$J_s$ measurements.** The Granier-type thermal dissipation probe (TDP) system was used to monitor $J_s$ of the inoculated trees and noninoculated, healthy trees 12 to 14 months after the multiple inoculations. In September 2009, sap flow monitoring was initiated on study trees in the Minnesota stand. Three 3-cm-long thermal dissipation probes (Dynamax Inc., Houston), each of which consisted of two sensors (heated and unheated serving as a reference), were radially inserted into the sapwood ≈30 cm above the uppermost inoculation heights around the stem of each tree. To prevent thermal interference, the heated sensor was installed 4 cm above the unheated sensor, as recommended by the manufacturer. Two probes were placed along two selected inoculation rows and one probe was placed between two inoculation rows. To provide waterproofing, the sapwood–air interface of each probe location was sealed with silicone and covered with a plastic cup. The stem area where three probes were placed was covered with reflective bubble wrap for thermal insulation. Signals from the sensors were monitored every 15 s, and 30-min means were recorded by a data logger (CR 10X; Campbell Scientific Inc., Logan, UT) for 18 days (18 September to 5 October). The signal recorded was the temperature difference between the heated and unheated sensors that was dependent on the rate of sap flow around the probes. As sap flow rates increased, heat was dissipated more rapidly and the temperature difference decreased (23).

$J_s$ in grams of $H_2O$ $m^{-2}$ $s^{-1}$ was calculated following Granier's equation (8) as $J_s = 119\ [(\Delta T_M - \Delta T)/\Delta T]^{1.231}$, where $\Delta T$ (°C) is the mean temperature difference between sensors during each half-hour measurement interval and $\Delta T_M$ is the maximum $\Delta T$ when there is no sap flow. Because sapwood depth (average of 2 cm) was shorter than the length of the 3-cm-long probe, the equation was calibrated for data obtained from the 3-cm-long probes following Lu et al (19). The software package BaseLiner (version 2.4.1; Hydro-Ecology Group, Duke University, Durham, NC) was used to calculate $\Delta T_M$ and $J_s$. $\Delta T_M$ was calculated as the maximum temperature difference measured over each 24-h cycle. Because this method can lead to an underestimate of nocturnal $J_s$ if water transport occurs at night, $\Delta T_M$ was considered eligible for data analysis only when there were no rain events within 24 h and the night-time vapor pressure deficit remained <0.2 kPa.

In July 2010, sap flow monitoring was initiated on study trees in the Wisconsin stand using one commercially manufactured (3-cm-long) (Dynamax Inc.) and two hand-manufactured probes on each tree. Sensors of a 2-cm-long probe were placed 10 cm apart according to Granier's (8) method. Probes were installed ≈30 cm above the uppermost inoculation heights along three vertical inoculation rows on the east, west, and north sides. Signals from the sensors were monitored every 15 s, and 30-min means were recorded by two data loggers (CR 23X; Campbell Scientific Inc.) for 24 days (30 July to 22 August).

**Evaluation of visual symptoms, pathogen recovery, and selected xylem properties.** Tree crowns were checked for symptoms of dieback or decline 12 or 13 months after inoculation but prior to sap flow monitoring. After sap flow monitoring was completed and TDP probes were removed, the size (length and width) of the canker (specifically, inner bark lesion) was measured for each inoculation point after the outer bark was removed using a drawknife. For Minnesota trees, the size of sapwood discoloration (length, width, and radial depth) beneath each canker was also measured after trees were felled and stem sections quartered using a bandsaw. For evaluating disease severity on each inoculated tree, the proportion of bark-cankered stem area (the inoculated stem section area with Ceratocystis cankers) was calculated by dividing total inner bark lesion area by the stem surface area where 50 inoculations were made. The area of each inner bark lesion was calculated using the equation for an ellipse [π × 1/2 (lesion length) × 1/2 (lesion width)]. Surface area of the stem section with 50 inoculation points was estimated using the equation for the outer surface area of a cylinder with the measured length (2 m plus length of longest inner bark lesion extending from top and bottom inoculation holes) and the average of the top and bottom diameters of the inoculated stem section [π × 1/2 (top diameter + bottom diameter) × (length)].

Blocks of stem tissue that contained the edges of four cankers or control wounds were excised from each tree and stored in polyethylene bags at 4°C. In the laboratory, small wood cubes were cut from the sampled blocks and placed in small moist chambers maintained at room temperature (≈25°C) to stimulate fungus sporulation. The wood cubes were examined at 10 and 20 days for presence of *C. smalleyi* perithecia. Ascospore masses on tips of perithecia found on the wood cubes were transferred to 2% malt yeast extract agar amended with streptomycin sulfate at 100 ppm to obtain fungal isolates.

Water transport within a plant largely depends on gradients in water potential along the soil–plant–atmosphere continuum and xylem structures that control hydraulic properties. Size of vessels is one of the important anatomical features of the xylem. According to the Hagen-Poiseuille law for volume flow through ideal capillary tubes, volume flow rate through a vessel increases with the fourth power of the vessel radius (26). Thus, hydraulic efficiency is governed by the proportion of large vessels that conduct more water. To determine whether there were any intrinsic differences in vessel size-related characteristics that could have affected water conduction in study trees, mean vessel diameter, mean hydraulic vessel diameter which gives more weight to large vessels, and the size distribution of vessels were measured. Two sapwood cubes (1.5 by 1.5 by 2.0 cm) were taken from the outer sapwood above and below each probe location. Cross sections (20 to 25 μm in thickness) that included the outer 10 growth rings were made from each wood cube using a sliding microtome (Model 860; American Optical, Southbridge, MA), stained in toluidine blue O (0.5% aqueous) and mounted in 10% glycerol. For each probe location, 500 vessels were analyzed (250 vessels for each wood cube; 50 vessels from the outer two growth rings and 200 vessels from the 3rd to 10th growth rings). To estimate vessel diameter (d), the maximum and minimum widths of each vessel were measured at ×100 magnification using imaging software NIS-Elements (Nikon, Japan) and averaged. Mean vessel diameter of each tree was calculated by averaging vessel diameters estimated in three probe locations. Mean hydraulic vessel diameter (D) (37) was also calculated from the measured vessel diameters (d) for each tree as $D = \Sigma d^5/\Sigma d^4$. For size distribution of vessels in an individual study tree, all measured vessel diameters were classified into 30-μm-diameter classes and the frequency of each diameter class was calculated.

A tylosis is a balloon-like evagination of protoplasm into a xylem vessel through a pit from an adjacent living parenchyma cell (1). Vessel occlusion by tyloses has been observed in trees with vascular wilt diseases such as oak wilt and Dutch elm disease (6,26). Because such extensive vascular plugging by tyloses has been closely linked to xylem dysfunction in trees with Japanese oak wilt and with sudden oak death (4,17,30), the abundance of tyloses formed in response to multiple *C. smalleyi* inoculations was evaluated. Tylose formation was observed at ×100 magnification with the same cross sections used for vessel diameter measurements. The proportion of vessels with tyloses among 500 vessels per probe location was estimated in each growth ring from the outermost to the 10-year-old growth ring ($n$ = 50 per growth ring). Tylose abundance in the outer two growth rings which might have more impact on water conduction and in the outer 10 growth rings was estimated separately.

**Data analyses.** Effects of *C. smalleyi* inoculation on symptom development (i.e., the average area of cankers) were compared with the inoculation wound area of water control trees using a two-sample $t$ test for means at 95% confidence level. Due to non-independence of the sensor-measured data, mean peak values of $J_s$ for each sap flow monitoring tree on a subset of days when monitoring was done ($n$ = 6, Minnesota; $n$ = 9, Wisconsin) were obtained from the cell means of a repeated-measures analysis of variance (ANOVA) output. Selection of the subset of days was chosen based on when representative trends of sap flow were detected for the full set of trees over the entire monitoring period (18 days, Minnesota; 24 days, Wisconsin). The resulting means and standard errors for $J_s$ for each control and fungus-inoculated tree were used to test for differences between the two treatment groups using two-sample $t$ tests for means at 95% confidence level. Mean vessel diameter, mean hydraulic vessel diameter, and tylose abundance (%) of fungus-inoculated trees were compared with those of control trees (water control and noninoculated trees) using a two-sample $t$ test for means at 95% confidence level. Factorial ANOVA was performed to determine whether the xylem properties were affected by location. A $\chi^2$ analysis was performed on the vessel diameter distribution to compare distributions in fungus-inoculated trees versus control trees for both the youngest 2 growth rings and the outer 10 growth rings. This was done to test whether inherent differences existed in the anatomy of the trees used for the two treatment groups. Correlation analysis using Pearson correlation coefficients was used to determine the significance of association between mean peak $J_s$ and proportion of

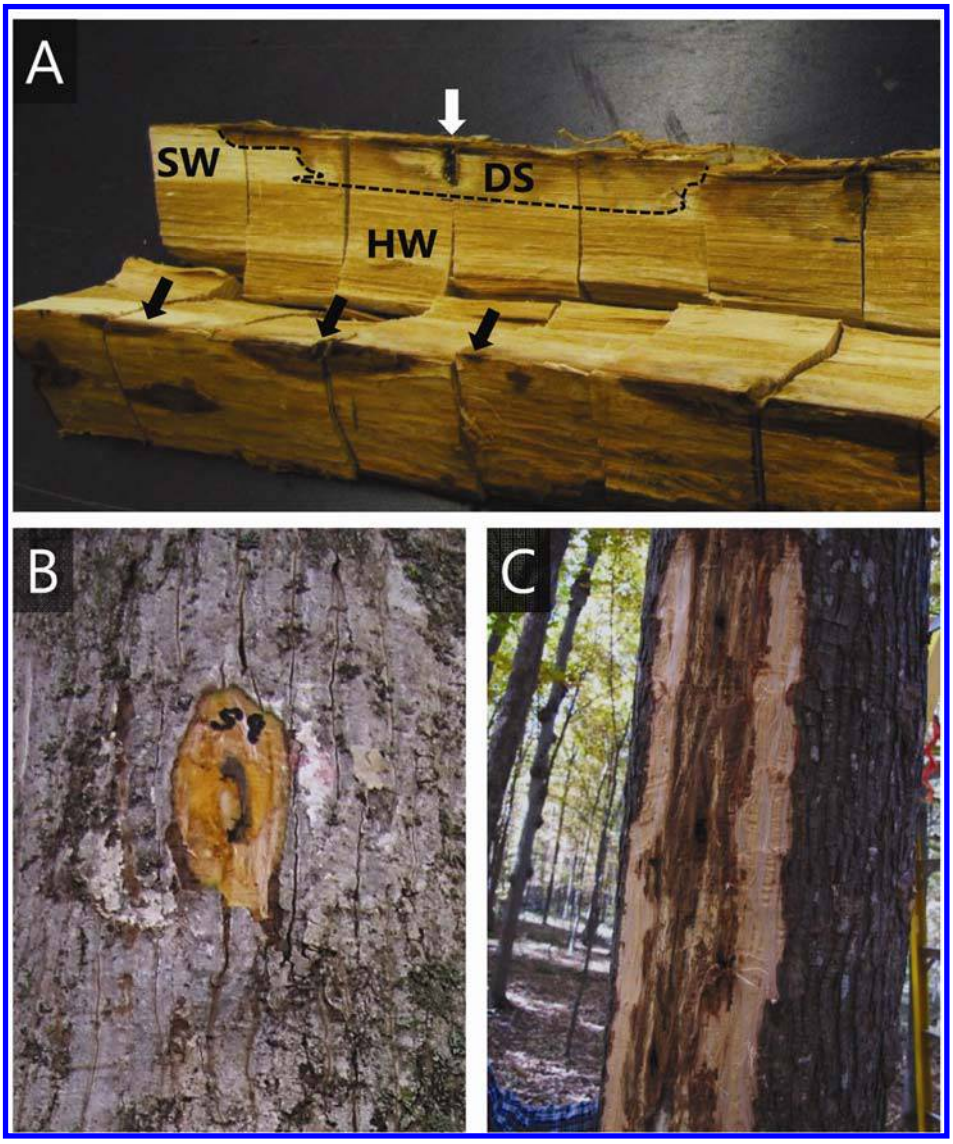


**Fig. 2.** Bark cankers and sapwood discoloration on bitternut hickory tree stems. **A,** Underlying sapwood discoloration (DS; delineated by dashed line) corresponding to three discontinuous bark lesions (black arrows) in longitudinal radial face of a *Ceratocystis smalleyi*-inoculated tree. White arrow indicates the inoculation point. SW: sapwood and HW: heartwood. **B,** Water-inoculated wound callused over by newly formed callus tissues on a water-inoculated tree. **C,** Diffuse cankers exhibiting long, reddish-brown inner bark lesions on one of the *C. smalleyi*-inoculated trees.

cankered or discolored stem area, mean vessel diameter, mean hydraulic vessel diameter, or tylose abundance measurements. When significant correlation was found, linear regression analysis was conducted to examine the relationship between mean peak $J_s$ and the measured parameter. All statistical analyses were conducted using SAS statistical software (version 9.1; SAS Institute Inc., Cary, NC). Numerical methods (the Kolmogrov-Smirnov test, Cramer-von Mises test, and Anderson-Darling test in the Univariate Procedure in SAS) for evaluating normal distribution and tests for equal variances (TTEST procedure in SAS) were conducted for data subsequently subjected to *t* test analysis. The Shapirio-Wilk W test was used to assess normality and the White test for equality of variances for data sets later subjected to linear regression analysis. When outliers were suspected, datasets were analyzed with and without the suspect points prior to the final analysis.

## RESULTS

**Visual disease symptoms.** Fourteen months after inoculation, no obvious external symptoms in the tree crown such as wilting leaves or crown decline were observed in any inoculated trees in Minnesota. In Wisconsin, only one of five fungus-inoculated trees exhibited a declining crown (≈60% visible dieback) 12 months after inoculation.

However, 12 to 14 months (Wisconsin and Minnesota, respectively) after the inoculation, stems of *C. smalleyi*-inoculated trees exhibited numerous cankers characterized by bark necrosis and sapwood discoloration (Fig. 2A) whereas no cankers were found on water-inoculated trees (Fig. 2B). The cankers were mostly long, narrow, diffuse cankers with reddish inner bark, as previously described (29) (Fig. 2C). Larger cankers (mean 64.3 ± 13.6 $cm^2$) developed in Wisconsin trees compared with those on Minnesota trees (mean 16.9 ± 3.0 $cm^2$), in spite of being evaluated 2 months earlier ($P < 0.0001$) (Fig. 3). In Wisconsin, many of the cankers coalesced (Fig. 2C). In such cases, lesion length was measured up to the middle point of the two inoculation points. This may have resulted in underestimation of canker area. For water controls, bark necrosis was restricted to the wound site itself and many inoculation points were callused over at the time of evaluation (Fig. 2B). Canker areas of *C. smalleyi*-inoculated trees were larger than necrotic areas associated with water control inoculation points ($\alpha = .05$).

The proportion of cankered stem area of fungus-inoculated trees was much larger than the proportion of discolored areas associated with water inoculation on control trees in both locations (Table 1). Furthermore, the proportion of cankered stem areas was consistently larger for fungus-inoculated trees in Wisconsin than in Minnesota.

For main stem sections from study trees in Minnesota, sapwood discoloration extended beyond its corresponding bark lesion along the stem axis when inoculated with the fungus (Fig. 2A). Occurrences of two to three discontinuous bark lesions corresponding to a continuous, discolored sapwood area were commonly observed in fungus-inoculated trees (Fig. 2A). Because many cankers had coalesced, it was hard to delineate discolored sapwood corresponding to an individual inoculation point. Thus, the length or volume of discolored sapwood was not measured. In the radial direction, sapwood was discolored up to 3.3 cm inward from the cambium in response to the fungal infection, reaching the outer boundary of heartwood in many cases. Control wounds had little, if any, discolored sapwood. *C. smalleyi* was recovered from all canker samples but was not isolated from the controls. Stem sections of Wisconsin study trees were not dissected to examine internal sapwood discoloration.

**Diurnal patterns of $J_s$.** All trees exhibited similar diurnal trends of sap flow (i.e., sap flow began to increase at approximately 6:00 a.m., continued to increase during the morning, peaked just after 12 noon, declined sharply after sunset, and remained low at night) (Fig. 4A and B). The values of $J_s$ did not differ between water-inoculated and noninoculated, healthy trees in the Wisconsin experiment (Fig. 4B). Compared with the control groups (pooled data of water-inoculated and noninoculated trees), fungus-inoculated trees had consistently lower flux of water during the midday hours at both sites (Fig. 4) ($\alpha = 0.05$).

To compare daily $J_s$ of fungus-inoculated trees to control trees 12 to 14 months (Wisconsin and Minnesota, respectively) after inoculation, peak values of $J_s$ on selected days were obtained from cell values of the output from repeated-measures ANOVA. Selected days were chosen when representative trends of sap flow were detected for the full set of trees. For control trees, mean peak $J_s$ was 26.3 to 30.0 g $m^{-2}$ $s^{-1}$ in Minnesota and 34.2 to 49.3 g $m^{-2}$ $s^{-1}$ in Wisconsin. Fungus-inoculated trees only achieved a mean peak $J_s$ of 10.7 to 18.1 g $m^{-2}$ $s^{-1}$ in Minnesota and 5.7 to 27.2 g $m^{-2}$ $s^{-1}$ in Wisconsin. In Minnesota, the mean peak $J_s$ of all infected trees (14.0 ± 2.1 g $m^{-2}$ $s^{-1}$) was reduced by 51% compared with control trees (28.6 ± 1.1 g $m^{-2}$ $s^{-1}$) ($P = 0.009$). In Wisconsin, inoculated trees experienced a 64% decrease in the mean peak $J_s$ (15.3 ± 3.8 g $m^{-2}$ $s^{-1}$) compared with control trees (41.9 ± 2.2 g $m^{-2}$ $s^{-1}$) ($P = 0.0001$). $J_s$ was consistently lower in Minnesota, where it was monitored later in the year (18 September to 5 October 2009) than in Wisconsin (monitored during the late summer, 30 July to 22 August 2010) (Fig. 4).

**Xylem properties of stems.** Vessel characteristics were analyzed to determine whether there were any inherent differences in tree anatomy that could have affected the hydraulic function of study trees. Because vessel size-related characteristics did not vary by site ($P = 0.17$ for vessel diameter and $P = 0.07$ for hydraulic vessel diameter), the data from both sites were pooled (Table 2). Mean vessel diameters and mean hydraulic vessel diameters did not significantly differ between control and fungus-inoculated trees ($P = 0.54$ and 0.19, respectively). One of five fungus-inoculated trees had relatively small mean vessel diameter (84.7 μm) and mean hydraulic vessel diameter (191.7 μm) in Wisconsin, thereby reducing the average values of the treatment group. Yet, differences in mean vessel diameters and mean hydraulic vessel diameters between fungus-inoculated versus control trees were insignificant. Vessel diameter distributions of control and inoculated trees were also homogeneous for the youngest two growth rings ($\chi^2 = 2.34$, $P = 0.99$) and for the outer 10 growth rings ($\chi^2 = 2.34$, $P > 0.99$) (Fig. 5).

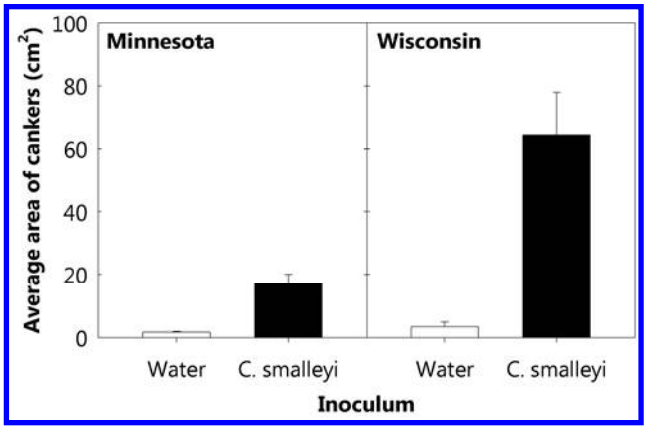


**Fig. 3.** Average area of cankers resulting from inoculation with four isolates of *Ceratocystis smalleyi* compared with area of discoloration associated with water inoculation on controls in two study sites. Canker development was evaluated 14 months after inoculation with two local isolates in Minnesota and 12 months after inoculation with two local isolates in Wisconsin. Measurements from 50 cankers or wounds of each study tree were averaged for the two isolates per site combined. Average values are presented for water-inoculated control trees ($n = 1$ in Minnesota and $n = 3$ in Wisconsin) and $n = 3$ or 5 trees for *C. smalleyi* inoculation in Minnesota and Wisconsin, respectively. Error bars represent standard error for canker or wound area.

Tyloses within vessels were observed in both control and fungus-inoculated trees but more frequently in the latter group (Table 2). In cross section, multiple tyloses were found to be occasionally produced from surrounding parenchyma cells into a vessel, thus completely occluding the vessel. Differences between the two treatment groups in tylose abundance were all significant in both the outer 10 growth rings and the outer 2 growth rings. The difference was more pronounced in the youngest two growth rings, where tyloses were formed at low frequency (average 9%) in the absence of fungal infection ($P = 0.0012$ in Minnesota and $P < 0.0001$ in Wisconsin). In control trees, tyloses were observed more frequently in vessels of the older sapwood than in vessels in the youngest two rings. This was expected because tylose production is a natural process that occurs as sapwood transitions to heartwood and ceases functional transport of water. Yet, fungus-inoculated trees had more vessels with tyloses in the outer 10 growth rings compared with those in the control trees ($P = 0.0059$ in Minnesota and $P < 0.0001$ in Wisconsin).

**Relationships between $J_s$ and disease severity or selected xylem properties.** Correlation analyses indicated that significant interactions were present between mean peak $J_s$ and proportion of cankered stem area ($P = 0.0042$ and $< 0.0001$ for Minnesota and Wisconsin, respectively), tylose abundance in the outer 2 growth rings ($P = 0.0084$ and $< 0.0001$ for Minnesota and Wisconsin, respectively), and tylose abundance in the outer 10 growth rings in Wisconsin but not Minnesota ($P = 0.1140$ and 0.0004 for Minnesota and Wisconsin, respectively), and between proportion of cankered stem area and tylose abundance in the two youngest rings ($P = 0.0045$ and $< 0.0001$ for Minnesota and Wisconsin, respectively) and tylose abundance in the outer 10 growth rings in Wisconsin but not Minnesota ($P = 0.1254$ and 0.0005 for Minnesota and Wisconsin, respectively).

Results of linear regression analyses for significant correlation results indicated that mean peak $J_s$ could be explained best by proportion of cankered stem area and tylose abundance in the youngest two growth rings in both sites (Fig. 6). Mean peak $J_s$ decreased as the proportion of cankered stem area increased (Fig. 6A and D). Similarly, mean peak $J_s$ decreased with greater tylose abundance in the youngest two growth rings (Figs. 6B and 6E). In Wisconsin, a decrease in mean peak $J_s$ was also significantly related to increased tylose abundance in the outer 10 growth rings (Fig. 6F).

## DISCUSSION

For both experiments combined, all but one study tree maintained healthy-appearing crowns up to the month of sap flow monitoring. The one Wisconsin tree that exhibited crown decline (60% in mid-July 2010) was fungus inoculated and had the highest proportion of cankered stem area (41.3%) of all the fungus- or water-inoculated trees on the site. The symptomatic tree also had the highest number of tyloses (74.7%) in the outer two growth rings compared with all other study trees. Several of the cankers were colonized by buprestid larvae (*Agrilus* sp.) that were revealed when the outer bark was peeled for canker measurement. None of the inoculation sites or resulting cankers on the other study trees was colonized by this or other insect pests. The lack of crown symptoms in the remaining trees, particularly fungus-inoculated ones, was not a surprise to the authors for several reasons. The number of fungus inoculation points used ($n = 50$)

TABLE 1. Characteristics of bitternut hickory trees used for sap flow monitoring study and proportion of cankered stem area 14 months after inoculation in Minnesota and 12 months after inoculation in Wisconsin

| Location, treatment, tree number[a] | Stem dbh (cm)[b] | Total inner bark lesion area ($cm^2$)[c] | Proportion of cankered stem area (%)[d] |
|---|---|---|---|
| Minnesota | | | |
| Fungus-inoculated trees | | | |
| 413 | 17.2 | 1,231 | 11.5 |
| 414 | 19.6 | 421 | 3.8 |
| 416 | 16.7 | 1,159 | 11.2 |
| Mean (SE) | 19.5 (0.90) | … | … |
| Water-inoculated tree | | | |
| 409 | 12.6 | 66 | 0.9 |
| Noninoculated trees | | | |
| 1 | 27.7 | NA | 0.0 |
| 2 | 18.2 | NA | 0.0 |
| Mean (SE) | 17.8 (4.41) | … | … |
| Wisconsin | | | |
| Fungus-inoculated trees | | | |
| 25 | 20.2 | 1,723 | 15.3 |
| 43 | 19.2 | 1,958 | 16.2 |
| 46 | 17.0 | 4,514 | 41.3 |
| 47 | 22.5 | 5,050 | 30.2 |
| 48 | 16.0 | 2,693 | 28.1 |
| Mean (SE) | 19.0 (1.16) | … | … |
| Water-inoculated trees | | | |
| 21 | 27.3 | 311 | 2.2 |
| 34 | 21.6 | 109 | 1.0 |
| 40 | 20.5 | 68 | 0.6 |
| Mean (SE) | 23.1 (2.10) | … | … |
| Noninoculated trees | | | |
| 29 | 25.5 | NA | 0.0 |
| 45 | 24.0 | NA | 0.0 |
| 49 | 26.0 | NA | 0.0 |
| Mean (SE) | 25.2 (0.60) | … | … |

[a] For mean and standard error (SE), data were averaged for one water-inoculated and two noninoculated, healthy trees for the control group.
[b] Diameter at 1.4 m of stem height.
[c] Total inner bark lesion area was the sum of 50 inner bark lesions (fungus-inoculated trees) and discoloration associated with inoculation wounds (water-inoculated trees) per tree. Each inner bark lesion area was calculated using the equation for an ellipse [π × ½ (lesion length) × ½ (lesion width)]; NA = not applicable.
[d] Proportion of cankered stem area was calculated by dividing total inner bark lesion area by the stem surface area where 50 inoculations were made on each tree using the equation for the surface area of a cylinder with known length, top diameter, and bottom diameter [π × 1/2 (top diameter + bottom diameter) × (length)]. The length was 2 m plus length of longest inner bark lesion extending from top and bottom inoculation holes.

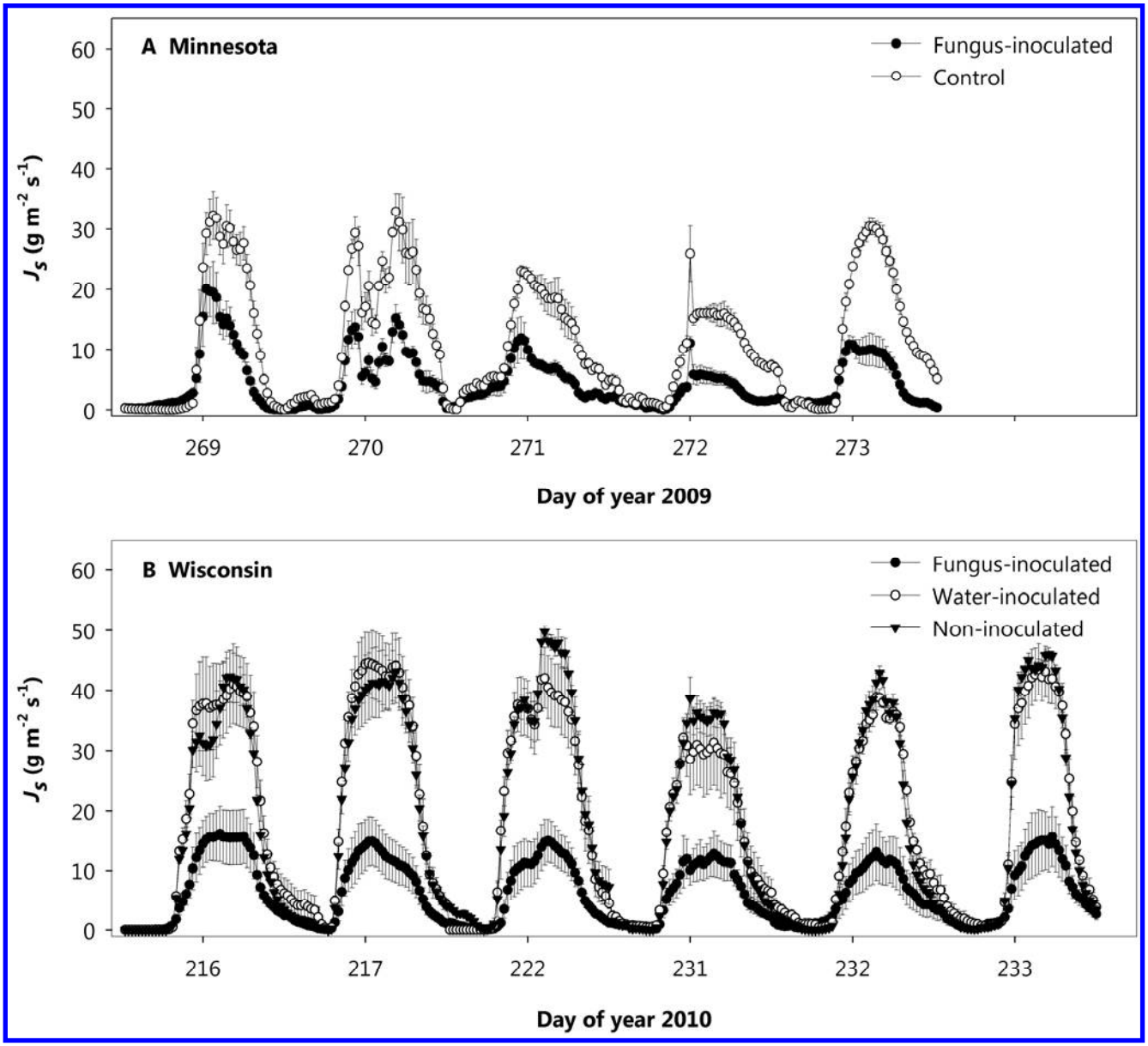


**Fig. 4.** Diurnal changes in sap flow velocity ($J_s$) in *Ceratocystis smalleyi*-inoculated trees versus water-inoculated and noninoculated controls on selected days during the study period. **A,** In Minnesota, data were averaged for three fungus-inoculated trees and one water-inoculated plus two noninoculated, healthy trees for a combined control group. **B,** In Wisconsin, data were averaged for five fungus-inoculated trees, three water-inoculated trees, and three noninoculated healthy trees for each treatment. Ticks in x-axis indicate noon on each day. Error bars indicate standard errors of the $J_s$ values for each treatment.

TABLE 2. Xylem properties of control and *Ceratocystis smalleyi*-inoculated trees[a]

| | Control[c] | | | *C. smalleyi*-inoculated | | | |
|---|---|---|---|---|---|---|---|
| Xylem properties[b] | *n* | Mean | SE | *n* | Mean | SE | *P* |
| Vessel characteristics (μm)[d] | | | | | | | |
| Mean VD | 9 | 103.3 | 8.9 | 8 | 101.3 | 2.8 | 0.54 |
| Mean hydraulic VD | 9 | 235.3 | 2.6 | 8 | 229.5 | 3.7 | 0.19 |
| Tylose abundance[e] | | | | | | | |
| Youngest 2 (%)[f] | | | | | | | |
| Minnesota | 9 | 8.9 | 2.6 | 9 | 30.4 | 4.8 | 0.0012 |
| Wisconsin | 18 | 8.8 | 1.5 | 14[f] | 56.0 | 5.4 | <0.0001 |
| Outer 10 (%) | | | | | | | |
| Minnesota | 9 | 24.5 | 2.2 | 9 | 36.5 | 3.1 | 0.0059 |
| Wisconsin | 18 | 42.4 | 2.0 | 14 | 58.9 | 2.5 | <0.0001 |

[a] SE = standard error.
[b] In all, 1,500 vessels were observed per tree.
[c] Data for control trees were pooled from water-inoculated trees and noninoculated trees.
[d] Data were pooled for nine control trees and eight fungus-inoculated trees from two locations because no statistical difference ($\alpha = 0.05$) was found by site; *n* = number of trees. VD = vessel diameter; hydraulic VDs were calculated as $\Sigma d^5/\Sigma d^4$ for each tree.
[e] Percent mean tylose abundance was determined for 500 vessels for each probe location, measured in youngest 2 and outer 10 growth rings; *n* = number of probe locations.
[f] Extensive colonization of vessels by fungus hyphae did not allow for tylose observation in 1 of 15 sapwood samples.

and that yielded cankers is generally lower than the number of cankers that have been observed on naturally affected bitternut hickory trees (11,13). The recent hickory bark beetle outbreaks in both stands had ended at least 2 years prior to initiation of the experiments. The inoculation points were <6 m height on the main stem and were not distributed evenly in a band around the tree. The majority of hickory bark beetle attacks and associated Ceratocystis cankers on actively declining bitternut hickory trees are generally found above 6 m in height on the main stem (J. Juzwik, *unpublished data*). Lastly, crown symptom development on trees within stands experiencing hickory bark beetle outbreaks generally occurs over 2 to 3 years. In monitoring five to six trees in each of four plots of such a stand in Shawano, County, WI, crown decline ratings in all but one tree progressed from <20% in July 2009 to 100% dead crown by September 2011 (J. Juzwik, *unpublished data*). The maximum length of time trees in this sap flow study were monitored (prior to destructive sampling) was 12 to 14 months.

The proportion of cankered stem area proved to be a good measure of disease severity. As previously mentioned, the only tree (fungus-inoculated) that exhibited crown decline symptoms had the highest proportion of bark with inner bark lesions compared with all the study trees. Furthermore, $J_s$ for all the fungus-inoculated trees was always lower than that for control (i.e., water-inoculated or the noninoculated trees in Wisconsin or the combined controls in Minnesota). Sap flow measurements of water-inoculated trees were similar to those of noninoculated control ones (Fig. 4B). The mechanical wounds associated with water inoculations had been closed by callus tissue when evaluated after 1 year. Thus, we hypothesize that multiple mechanical damage alone, such as hickory bark beetle damage without *C. smalleyi* infections, is not likely the cause of rapid crown decline in bitternut hickory. In contrast, the reduced hydraulic function observed in trees artificially inoculated with *C. smalleyi* suggests that multiple *C. smalleyi* infections and accompanying host sapwood responses impede water transport in affected trees. Reduced $J_s$ associated with sapwood colonization by pathogens has been documented for *R. quercivora* in *Quercus* spp. and *P. ramorum* in tanoak (18,30).

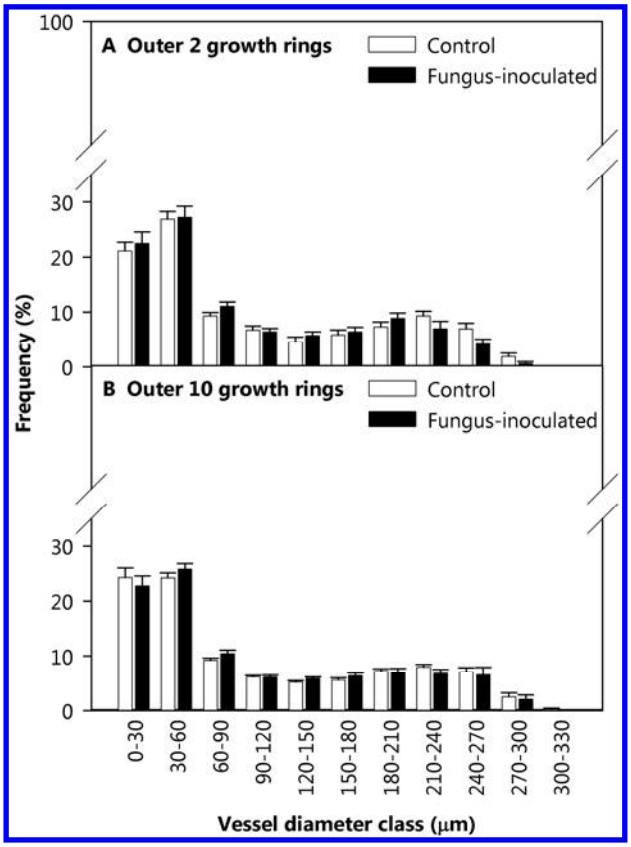


**Fig. 5.** Distribution of vessel diameters for control and fungus-inoculated trees. **A,** Vessel diameter distribution from the youngest two growth rings. Data shown were pooled results of the Minnesota and Wisconsin sites (nine trees for control and eight trees for fungus-inoculated group, 300 vessels per tree were evaluated). **B,** Vessel diameter distribution from the outer 10 growth rings. Data shown were pooled results of the Minnesota and Wisconsin sites (nine trees for control and eight trees for fungus-inoculated group, 1,000 to 1,500 vessels per tree were evaluated). Error bars represent standard error for vessel diameter frequency. The $\chi^2$ statistics showed no difference in inherent vessel diameter distribution of fungus-inoculated trees versus control trees ($\chi^2 = 2.34$, $P = 0.99$ for the youngest 2 growth rings and $\chi^2 = 0.54$, $P > 0.99$ for the outer 10 growth rings).

Rapid crown decline symptoms can be logically explained by reduced water flow. However, correlation between reduced sap flow and crown dieback or death was not an objective of our hickory field studies. However, such a study would be possible using the same sap flow monitoring system. Sap flow was monitored in Norway spruce mass inoculated with *C. polonica* from 3 weeks prior to inoculation to 5 months after inoculation (15). Dramatic reduction in sap flow was followed by no sap flow 4 to 6 weeks after spruce inoculation. Obvious external symptoms and tree death were observed the next spring (i.e., just <1 year after fungus inoculation) at 403 to 416 points on a 1.2-m-wide band on the lower stem of each tree. If such a study were conducted in bitternut hickory, a minimum of 100 inoculations with *C. smalleyi* starting at 6 m of stem height and extending higher would be needed to mimic natural infections on actively declining trees. Furthermore, the inoculations ideally would be scattered around the circumference of the stem to simulate the spatially dispersed nature of hickory bark beetle attacks. An aerial lift capable of working in the uneven terrain of a forest stand would be required for both inoculation and sap flow monitoring in such a study. A sap flow monitoring study in a naturally affected, actively declining tree could be conducted over time but ongoing attacks by the hickory bark beetle and, possibly, other biotic agents besides *C. smalleyi* could prevent direct correlation between fungus infection and tree death.

Vessel diameter and vessel diameter distribution in the outer 2 and the outer 10 growth rings did not differ for fungus-inoculated versus control treatment. This evidence supports the assertion that reduced $J_s$ in the fungus-inoculated trees was not a result of their inherent xylem properties. *C. smalleyi* inoculations were correlated with significantly more vessels plugged by tyloses compared with that in water-inoculated and noninoculated control trees. The higher incidence of tyloses in infected trees versus control trees is not a surprising result because tylose formation is a well-known host response to colonization by a xylem pathogen (3). Tyloses are known to form in response to xylem cavitation, which can be induced by pathogens that may physically block a xylem vessel or may secrete substances that alter physical properties of sap within a vessel (16,36). Tylose development also has been found to occur in response to increased ethylene production in grapevine stems that is stimulated by wounding or pathogen colonization (35). Vessel occlusion by such tyloses has been commonly observed in trees with vascular wilt diseases such as oak wilt and Dutch elm disease (3,6,26). *P. ramorum*, the sudden oak death pathogen, also was found to induce tylose formation in tanoak sapwood (4,30).

Of the selected xylem properties considered in our study, only tylose abundance in the youngest two growth rings was significantly and inversely correlated with $J_s$ in trees on both sites. Tyloses increase resistance to flow of water by reducing the radius of a vessel and may completely occlude the vessel when the tyloses are large or abundant (5). Limited distribution or few tyloses may have little effect on total water transport because, when an individual vessel ceases to function, water can flow around the affected vessel through pit openings into adjacent functioning vessels and continue to move to distal tissues (26). However, ex-

tensive occlusion of vessels by tyloses can affect sustained hydraulic transport of trees by dehydration (26). Furthermore, such extensive blockage of xylem vessels increases tension in the remaining vessels to maintain water flow to tree crowns, resulting in increased occurrences of embolism and increasingly reduced hydraulic conductivity (36). Using sap flow and hydraulic conductivity measurements, reduced hydraulic function of xylem in relation to tylose abundance has been documented in tanoak trees either naturally infected or artificially inoculated with *P. ramorum* (4,30). Therefore, tylose formation associated with *C. smalleyi* infection, particularly in youngest growth rings where the most volume of water is transported, is likely responsible for much of the water transport disruption found in our study. Hickory trees are ring porous; therefore, blockage of large-diameter vessels produced in springwood would exacerbate the impact on water flow. In a separate histological study, Park (27) observed accumulation of gels in vessels as well as tyloses at the margin of discolored sapwood in *C. smalleyi*-inoculated bitternut hickory. Thus, gels also may have contributed to reduced sap flow in our study.

Infection of sapwood by *C. smalleyi* suggests additional symptomology and pathogenesis in affected bitternut hickory trees. Numerous sapwood infections by the fungus are likely required for impaired sap flow to occur because spores of the fungus have not been observed to move systemically throughout an inoculated tree (27). The reduced sap flow resulting from multiple infections is a plausible hypothesis for wilting observed in rapidly declining crowns of affected trees. If additional field studies were conducted and numerous infections were found to correlate with crown decline, *C. smalleyi* could be considered to be a limited vascular wilt pathogen as well as a bark canker pathogen on bitternut hickory. Parke et al. (30) concluded that *P. ramorum* can be a vascular wilt pathogen on tanoak based on their histological and physiological studies of that pathosystem.

We hypothesize that hickory bark beetle damage without accompanying *C. smalleyi* infection is not sufficient to cause the rapid crown decline symptoms observed in forest stands experiencing disease outbreaks. Because Ceratocystis cankers accompanying hickory bark beetle galleries commonly extended beyond the limits of the insect's damage on stems of bitternut hickory with actively declining crowns, girdling of the cambium by tunneling activity of the pest does not occur early enough in development of the disease to cause tree death (9,24,33). We propose that the interaction of the two biotic agents is needed for disease progression and tree death to occur. Based on frequent isolation of the pathogen from hickory bark beetles initiating attacks on bitternut hickory in late summer (11,13), it is apparent that the beetle provides an entry for the fungus to infect the tree. Multiple infections caused by the fungus are then able to disrupt water transport in the affected trees. Thus, numerous bark cankers associated with thousands of hickory bark beetle attacks, numerous sapwood infections underlying the cankers, and fungus-induced anatomical changes in the sapwood correlated with reduced $J_s$ constitute one plausible explanation for the rapid crown decline symptomology observed in nature.

In conclusion, our study showed that multiple infections of *C. smalleyi* affected physiological function involving water transport in maturing bitternut hickory. Disease severity, as measured by proportion of cankered stem area, was associated with the reduced velocity of sap flow in diseased trees. Tylose abundance

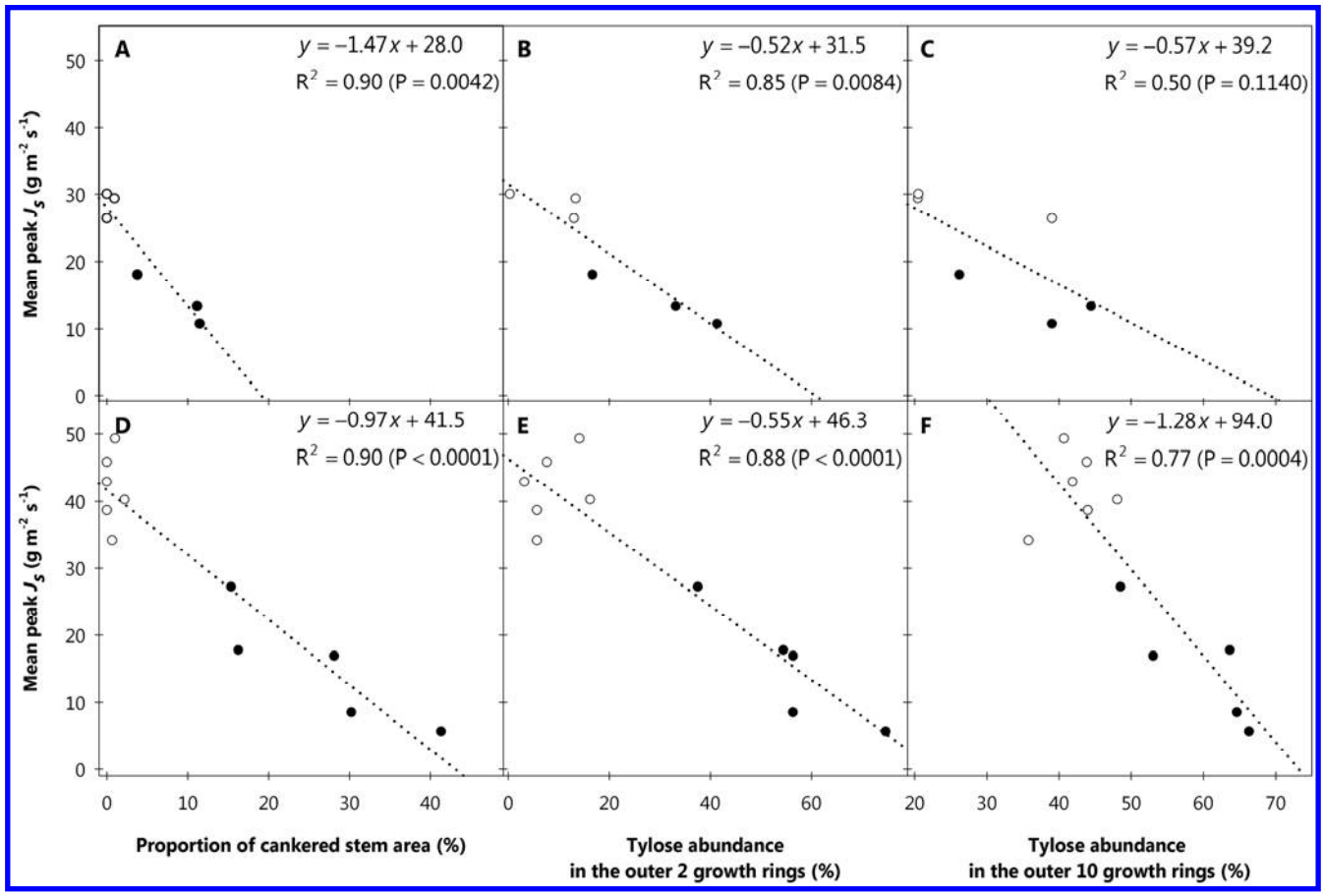


**Fig. 6.** Linear regression of mean peak sap flow velocity ($J_s$) versus proportion of cankered stem area and tylose abundance with different sites shown separately; **A to C,** Minnesota and **D to F,** Wisconsin. Proportion of cankered stem area was calculated by dividing total inner bark lesion area by the stem surface area where 50 inoculations were made on each tree using the equation for the surface area of a cylinder with known length, top diameter, and bottom diameter [π × 1/2(top diameter + bottom diameter) × (length)]. The length was 2 m plus the length of longest inner bark lesion extending from top and bottom inoculation holes. Open circles denote control trees and closed circles denote fungus-inoculated trees.

in current and preceding year's vessels associated with *C. smalleyi* infections is likely responsible for much of the water transport disruption. Further study is needed to test the hypothesis that multiple main stem infections of *C. smalleyi* can cause rapid crown decline of bitternut hickory.

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