BIOTIC AGENTS RESPONSIBLE FOR RAPID CROWN DECLINE AND MORTALITY OF HICKORY IN NORTHEASTERN AND NORTH CENTRAL USA

Jennifer Juzwik, Ji-Hyun Park, Mark T. Banik, and Linda Haugen[1]

Abstract.—Severe decline and mortality of hickory (*Carya* spp.) occur periodically in the eastern United States. Recently, rapidly declining crowns followed by tree mortality were found to be the predominant symptoms based on a 2 year survey in six north central and northeastern states. Stems of actively declining bitternut hickory (*Carya cordiformis*) exhibited numerous cankers and evidence of hickory bark beetle (*Scolytus quadrispinosus*) (Hbb) colonization. Main stems of three affected trees (40 to 80 percent crown decline) had 178 to 1,448 bark beetle attacks. *Ceratocystis smalleyi*-caused cankers and xylem lesions were associated with 13 to 37 percent of these attacks. The fungus was commonly isolated from adult Hbb attacking bitternut hickory in late summer, but not from Hbb emerged from declining trees in early summer. Fifty *C. smalleyi* inoculations (2 to 4 m stem height) of eight bitternut hickory (13 to 28 cm diameter at breast height [d.b.h.]) resulted in reduced sap flow rates (35 to 86 percent) 12 to 14 months after treatment compared to nine controls. More xylem vessels were occluded by tyloses in inoculated trees compared to controls. These results suggest that multiple cankers and xylem dysfunction caused by *C. smalleyi* are likely major contributors to crown decline. The disease is apparently a result of the synergistic interaction of Hbb and the pathogen.

INTRODUCTION

Hickory decline, particularly of bitternut hickory (*Carya cordiformis*) and shagbark hickory (*C. ovata*), to a lesser extent, has recently been noted in Iowa (Johnson et al. 2005), in Missouri, Maryland, New York, Pennsylvania, and West Virginia by Forest Health Monitoring (Steinman 2004), and in Wisconsin (Wisconsin DNR 2005). A Forest Service-led project was initiated in 2007 with funding from the Forest Health Evaluation Monitoring Program to elucidate cause(s) of the widespread problem (U.S. Forest Service 2011). Based on a 2007-2008 field survey conducted in six states as part of the project, the most common hickory health problem observed in affected stands involved rapidly declining crowns of smooth-bark hickories (bitternut and pignut [*C. glabra*], in particular) over 1 to 2 years followed by tree death (Juzwik et al. 2009).

Historically, episodes of hickory mortality have occurred periodically since the early part of the 20th century. At that time, serious mortality of the species was documented in much of their natural range from Wisconsin to Vermont and south to central Georgia (Hopkins 1912). Within the first decade of that century, thousands of hickories died in central New York State alone (New York State Museum 1910). Subsequent periodic episodes were observed and reported through the rest of the century (U.S. Forest Service 1985). In Wisconsin, for example, episodes of hickory decline or dieback have been reported in the late 1960s, late 1980s, and early 2000s (U.S. Forest Service 1994, Wisconsin DNR 2005).

[1]Research Plant Pathologist (JJ), U.S. Forest Service, Northern Research Station, 1561 Lindig St., St. Paul, MN 55108; Postdoctoral Scientist (J-HP), Korea Forest Research Institute, Seoul, Republic of Korea; Microbiologist (MTB), U.S. Forest Service, Northern Research Station; and Plant Pathologist (LH), U.S. Forest Service, Northeastern Area State and Private Forestry. JJ is corresponding author: to contact, call 651-649-5114 or email at jjuzwik@fs.fed.us.

Widespread mortality of hickory has historically been attributed to outbreaks of the hickory bark beetle (*Scolytus quadrispinosus*) during extended periods of drought (U.S. Forest Service 1985). The insect is considered the most important pest of hickory species (Solomon and Payne 1986). In 1994, a newly discovered fungus was reported in discolored wood and sunken bark cankers associated with beetle attack*s* (U.S. Forest Service 1994*).* This fungus, *Ceratocystis smalleyi,* and a related species, *C. caryae*, were recently described (Johnson et al. 2005). Both species were pathogenic on 2-year-old *Carya* spp. in greenhouse studies. The researchers suggested that *C. smalleyi* might play a significant role in hickory mortality. Subsequent field inoculations on pole-timber sized bitternut hickory in Iowa and Minnesota documented the ability of *C. smalleyi* to cause large bark cankers and xylem lesions on the species within 12 to 14 months after inoculation (Park et al. 2010).

Field and laboratory studies were conducted between 2008 and 2011 to investigate the nature of the interaction between the hickory bark beetle and *C. smalleyi* on stems of bitternut hickory with actively declining crowns and to determine the role of *C. smalleyi* in rapid crown decline and mortality in this species. Specific objectives for the fungus–bark beetle studies were to: 1) document the frequencies of occurrence of *C. smalleyi* cankers and hickory bark beetle attacks on bitternut hickory with actively declining crowns, and 2) determine the frequencies of hickory bark beetles carrying propagules of *C. smalleyi* when initially attacking bitternut hickory and when emerging from colonized trees. Specific objectives for the studies focused on the pathogen were to: 1) document sap flow rates in multiple-cankered bitternut hickory versus canker-free trees, 2) explore relationships between sap flow rates and extent of stem cankering as well as induced host responses that are known to reduce xylem water flow, and 3) document extent of pathogen colonization and host response in the xylem of artificially inoculated bitternut hickory. This paper proposes Ceratocystis wilt of hickory caused by an interaction of *C. smalleyi* and the hickory bark beetle as a new disease that is hypotheized to cause crown decline and tree mortality.

STUDY AREAS

In general, forest stands of 4 to 89 ha with different levels of hickory stocking were used for the field studies. Crown decline and mortality of bitternut hickory were common in the stands. The field site in Minnesota (Wabasha County) was in a mixed hardwood stand (4 ha) with pole-timber size (13 to 28 cm d.b.h.) bitternut hickory accounting for 40 percent of the stand. Field studies were conducted at three sites in Wisconsin. The mixed hardwood stand (8 ha) used in Chippewa County, WI, was thinned during winter 2007-2008. In 2009 and 2010, bitternut hickory made up 20 to 25 percent of the stand and was mostly pole-timber size. A privately-owned mixed hardwood stand (32 ha) with 25 percent bitternut hickory in Marathon County, WI, included both pole and sawtimber size (> 28 cm d.b.h.) bitternut hickory. The Shawano County, WI, stand was located on Stockbridge-Munsee Community lands and consisted of 89 ha of mixed hardwoods with 25 percent bitternut hickory, primarily >28 cm d.b.h.

MATERIALS AND METHODS

Debarking Stems of Trees with Declining Crowns

Two bitternut hickory with 40 and 55 percent crown decline in Marathon County and one with 80 percent decline in Chippewa County were felled in June 2009. The main stems of the trees (21 to 23 m tall) were cut into approximately one meter long sections, wrapped in insect screening,

and transported to the laboratory. The screened logs were stored at 4 ºC until processed. The bark was stripped from each section using a drawknife. The presence and extent of stem attack and colonization by hickory bark beetles were recorded for each section. In addition, the numbers of visible xylem lesions typical of Ceratocystis canker of hickory (Park et al. 2010) were determined and any association with bark beetle attacks recorded.

Collection and Bioassay of Bark Beetles

Attacking bark beetles

Initial hickory bark beetle attacks were detected on bitternut hickory with actively declining crowns in Minnesota and two of the Wisconsin (Marathon and Shawano County) field sites. Between 26 August and 2 September 2009, five of six trees were felled to facilitate collection of bark beetle adults as they were constructing entry tunnels; ladders were used to collect from the sixth tree. The outer bark was carefully scraped around the entry hole and needle probes were used to gently push each individual adult beetle from its entry tunnel and into a 1.5 ml micro-centrifuge tube. Captured bark beetles were grouped by source tree number and geographical location and stored on ice for transport to the laboratory where beetles were sexed and then stored at -10 ºC until further processed.

Emerging Bark Beetles

Based on prior experience and the literature (Goeden and Norris 1964), hickory bark beetles were known to start emerging from colonized bitternut hickory in mid to late June in Wisconsin. In order to sample colonized trees prior to emergence, actively declining trees were identified in mid-June of each collection year. In 2010, two trees were felled and stems examined for entry holes of the beetle from late summer 2009 attacks. A drawknife was used to expose galleries associated with holes and any adults beetles were carefully removed with insect forceps. These captured, "excavated" beetles were stored as previously described for attacking bark beetles. In 2009, three actively declining trees in Shawano Co., WI, and four in Marathon County, WI, were felled and stems closely examined for evidence of hickory bark beetle activity. Two to three 0.7-m-long stem sections were removed from colonized portions of each tree, each section was wrapped and sealed in insect screening, and all were transported to the laboratory. Each stem section was placed into a 1 m long by 38 cm diameter cardboard tube with aluminum screening on the outside and the ends sealed with plastic caps. Emerged bark beetles were obtained from the collection cup protruding from the side of the tube. Collections were made twice per week until no further adult emergence occurred. The collected bark beetles were counted, sex identified, and stored singly in micro-centrifuge tubes as previously described.

Bioassay of Bark Beetles

Bark beetles were assayed for the presence *C. smalleyi* on their exoskeleton using either serial dilution plating techniques or polymerase chain reaction (PCR) and cloning techniques. Serial dilution plating detects only the viable fungus propagules present while the PCR method amplifies DNA of living and dead fungi that are present. Sterilized water (0.5 ml) was added to each microcentrifuge tube containing a single bark beetle, and the suspension was subjected to sonication (10 seconds) using a tip sonicator (Ultrasonic Homogenizer, Cole-Palmer, Vernon Hills, IL) to dislodge fungal propagules present on the exoskeleton of each bark beetle. The resulting solution was then serially diluted (3X), and 0.5 ml aliquots of each dilution were spread onto 2 percent malt yeast extract agar amended with 100 ppm streptomycin sulfate in petri dishes. The plates were stored in an incubator

(dark conditions, 24 ºC) for 14 days. The number of *C. smalleyi* colonies were then determined and recorded. Identification of the fungus was based on culture morphology, presence of perithecia and extruding ascospores, and characteristic endoconidia (Johnson et al. 2005). All bark beetles were processed within 3 months of capture. PCR and cloning were used to detect the presence of viable or nonviable propagules of the fungus on a different subset of beetles from the attacking and emerged beetle collections. The fungal DNA associated with 26 attacking and 21 emerged bark beetles was extracted. Each bark beetle was transferred from its storage tube to a sterile 1.5 mL centrifuge tube, 200 µL of cell lysis buffer (Lindner and Banik 2009) was added, and each tube was sonicated as previously described. The tubes were then heated at 65 ºC for 1 hour, centrifuged at 10,000 g for 5 min, and 100 µL of the supernatant was removed to a 200 µL strip-tube. The DNA from each sample was then precipitated with isopropanol and cleaned with glass milk (Lindner and Banik 2009). The cleaned DNA was suspended in 50 µL molecular grade water and used as template DNA for PCR. The ITS (internal transcribed spacer) region of the DNA was amplified using the fungal specific primer pair ITS1F and ITS4. The PCR reaction protocol, thermal cycler parameters, and cloning methodology used were those of Lindner and Banik (2009). Either 8 or 16 bacterial colonies which had been successfully transformed with PCR products were re-amplified in PCR as before. The resulting PCR products were diluted approximately 1:10 with molecular grade water and cycle sequenced per methods in Lindner and Banik (2009). The resulting sequences were compared against known *C. smalleyi* ITS sequences using Sequencher® version 5.0 sequence analysis software (Gene Codes Corp., Ann Arbor, MI).

Hickory Inoculation and Canker Evaluation for Sap Flow Study

Fungus Inoculation

To mimic the natural occurrence of multiple cankers on bitternut hickory, multiple sites on stems of each study tree (between 13 and 28 cm d.b.h.) were inoculated with *C. smalleyi*. In July 2008, 50 holes (0.6 cm diameter) were made by drilling into the outer sapwood on the main stem of nine healthy bitternut hickory trees between 1.8 and 3.7 m stem height in Wabasha County, MN. The holes were mostly situated in four longitudinal lines, one on each cardinal aspect of the tree. Aliquots (0.1 ml) of *C. smalleyi* spore suspensions (1.0×10^4 ascospores/ml) of two locally-derived isolates and of sterile distilled water (control) were placed into the drilled holes (Park et al. 2010). Each hole was sealed with moist cotton and moldable putty. Noninoculated trees (three) served as negative controls. In July 2009, the same methods were used to repeat the experiment in Chippewa County, WI.

Canker Evaluation

Stems of the fungus and water inoculated trees were examined for presence of visible cankers 12 to 14 months after treatment. Once sap flow measurements were completed, the bark around each inoculation hole was stripped using a drawknife, and the extent of inner bark necrosis was recorded. Using the estimated area of each canker and total area of the bark for the 1.9 m long stem section receiving treatments, the proportion of the total stem area with cankered tissues was calculated for each tree. Wood samples that contained the edges of four cankers or inoculation wounds (for water controls) were taken from each tree to verify the presence of *C. smalleyi.* Small wood cubes were cut from each sample and placed in small moist chambers to stimulate fungus sporulation. Ascospore masses exuded from perithecia formed on the cubes were transferred to 2 percent malt yeast extract agar amended with 100 ppm streptomycin sulfate to obtain fungal isolates.

Measuring Sap Flow Rates

Granier-type thermal dissipation probes (TDP) and their associated system were used to monitor sap flow rate (Js) of each study tree (inoculated and noninoculated). Using these techniques, reduced sap flow velocity has been observed with sudden oak death in tanoak (*Lithocarpus densiflorus*) (Parke et al. 2007) and transpiration in deciduous broadleaf trees monitored in a suburban landscape (Peters et al. 2010). Each probe consists of a heated and an unheated sensor, and the signal recorded is the temperature difference between the two sensors. To prevent thermal interference, the heated sensor was installed 4 cm above the unheated sensor as recommended by the manufacturer (Dynamax Inc., Houston, TX). The temperature difference is dependent on the rate of sap flow around the probes. As sap flow rates increase, heat is dissipated more rapidly and the temperature differences decrease (Smith and Allen 1996).

For the Minnesota experiment, probes were installed on four fungus-inoculated, one water-inoculated, and two noninoculated trees in mid-September 2009. Three 3-cm-long manufactured probes (Dynamax Inc., Houston, TX) were inserted radially into the sapwood approximately 30 cm above the uppermost inoculation points on each tree stem. Two probes were located above two of the inoculation "columns" while the third was placed between the two columns. To prevent rain water from reaching the probes, silicone was applied to the probe-sapwood interface, and a plastic cup covered each sealed probe. Reflective bubble wrap applied around the stem at probe height provided thermal insulation. Signals from the sensors were monitored every 15 seconds, and 30 minute means were recorded by a data logger (CR 10X, Campbell Scientific, Inc., Logan, UT) for 18 days. For the Wisconsin experiment, probes were installed on five fungus-inoculated, three water-inoculated, and three noninoculated trees in late July 2010. However, two 2-cm-long, hand-made probes and one 3 cm long manufactured probe were used for each tree. As in Minnesota, the probes were placed 30 cm above the uppermost inoculation points, but in Wisconsin all three probes were placed above inoculation columns.

The sap flow rate, J_s in g H_2O m^{-2} s^{-1}, was calculated according to Granier's equation (Granier1987):

$$J_s = 119\left(\frac{\Delta T_M - \Delta T}{\Delta T}\right)^{1.231}$$

Where ΔT (°C) is the mean temperature difference between sensors during each half-hour measurement interval. The value of ΔT_M is determined when ΔT_M is at the peak over each 24 h cycle.

The software package BaseLiner (version 2.4.1, Hydro-Ecology Group, Duke University, Durham, NC) was used to calculate ΔT_M and J_s . The equation was calibrated in case the sapwood depth is shorter than the probe length (Lu et al. 2004).

Canker Development Evaluation and Sampling for Colonization Study

Fungus Inoculation and Temporal Sampling

Two locally derived isolates of *C. smalleyi* were grown on 2 percent malt yeast extract agar. and extruded masses of ascospores were transferred from tips of perithecia to 1.0 ml sterile distilled water. The suspension was homogenized using a tip sonicator, and the resultant suspension was adjusted to

a concentration of 1×10^4 ascospores/ml. In late June 2008, four holes (6 mm diameter) were drilled into the outer sapwood around the circumference of each tree, and 0.1 ml of the inoculum was placed into each hole. Moist cotton and masking tape was used to cover the inoculated holes. Four *C. smalleyi* inoculated and two sterile water inoculated tree were evaluated at 2 months postinoculation; two *C. smalleyi* inoculated trees only were evaluated at 12 months. There were four inoculation points per tree in all cases. Stem sections (1.0 m in length) containing the four inoculated points were cut from the inoculated trees at 2 and at 12 months, wrapped and sealed for transport to the laboratory, and stored at 4 °C until further processed.

Sample Processing

Each stem section was examined for symptoms of diffuse bark cankers and xylem lesions/ discoloration. A drawknife was used to remove the outer bark so inner bark lesions could be measured. Using a band saw, sections were then quartered along the longitudinal axis to separate each canker or control wound. Length of discolored sapwood associated with each inoculation point was then recorded. Stem slices were removed from the longitudinal ends of xylem lesions resulting from the inoculations. Small wood cubes were cut from these slices and attempts made to re-isolate the fungus (as previously described). Subsamples were also taken for histological study. Three 2-cm thick wood slices were cut at 2, 12, and 22 cm from each inoculation point. Wood cubes (1.5 cm by 1.5 cm by 2 cm) were subsequently obtained from the interface between clear and discolored sapwood of these wood slices and fixed in formaldehyde: acetic acid: ethanol solution prior to sectioning.

Histological Evaluations of Sapwood

Observations and Measurements for Sap Flow Study

Three measurements (mean vessel diameter, mean hydraulic vessel diameter, and size distribution of vessels) were made to determine if any intrinsic differences in vessel sizes existed that could have affected water conduction in study the trees. Two sapwood cubes (1.5 cm by 1.5cm by 2.0 cm) were taken from above and below each probe location. Cross sections (20-25 µm) were collected from each wood cube using a sliding microtome (Model 860, American Optical, Southbridge, MA), were stained in toluidine blue O (0.5 percent aqueous), and were mounted in 10 percent glycerol. Five hundred vessels were analyzed for each problem location (250 vessels for each wood cube). Vessel diameters were measured at 100x magnification using imaging software (NIS-Elements, Nikon Instruments Inc., Melville, NY). Mean diameters for treatment group was calculated by averaging vessel diameters obtained for every probe location on trees within the group. Mean hydraulic vessel diameter also was calculated for each treatment as $\sum d^5/\sum d^4$. Frequency distributions of vessel diameters were based on 30 um diameter classes using measurements for 500 vessels for each probe location within each treatment group. Tylose formation was observed at 100x magnification with the same cross sections used for vessel diameter measurements. The number of vessels with and without tyloses in each annual ring from the current-year ring back to the 9th or 10th ring from the current-year ring was counted (n = 500 per probe location).

Observations and Measurements for Fungus Colonization Study

Of the two wood cubes obtained from sampled points at increasing distance from the inoculation point, one was sectioned transversely and the other longitudinally to a 20 to 25 um thickness using a sliding microtome. The occurrences of fungal hyphae, tyloses, and gels in vessels were observed by

light microscopy of transverse sections stained with toluidine blue O mounted in 10 percent glycerol. For each section, 250 vessels were examined and numbers recorded for: 1) occluded vessels with tyloses or gels, 2) nonoccluded but fungus-colonized vessels, and 3) nonoccluded, sound vessels.

Data Analyses

For both fungus colonization and sap flow studies, one-way analysis of variance (ANOVA) was used to test for differences between inoculum type and the average size of inner bark cankers. When the F-statistic was significant, differences in means were determined using Tukey's HSD ($\alpha = 0.05$). The averages of maximum sap flow rate measured on multiple days were estimated for each tree by repeated measures ANOVA. Two-sample t-tests (at 95 percent confidence level) were used to compare mean maximum flow rate, mean vessel diameter, mean hydraulic vessel diameter, and tylose abundance to those of control trees. Pearson's chi-square statistics ($\alpha = 0.05$) were used to detect significant correlation among mean maximum sap flow rate, proportion of cankered bark, and selected xylem features. Linear regression analyses also were used to investigate relationships between these same variables. For the fungus colonization study, mean percentages of occluded, fungus-colonized, and sound vessels of fungus inoculated trees 2 and 12 months after inoculation with *C. smalleyi* or sterile water were compared using two sample t-tests for means (95 percent confidence level). All statistical analyses were conducted using SAS 9.1 (SAS Institute, Inc. Cary, NC).

RESULTS

Hickory Bark Beetle-*Ceratocystis smalleyi* Association on Declining Trees

Hickory Bark Beetle Attacks and Canker Occurrence

Debarking of the main stems of three bitternut hickories exhibiting active crown decline revealed attacks by hickory bark beetles characterized by aborted to fully successful colonization (i.e., full gallery system) (Table 1). No clear relationship was observed between total number of bark beetle attacks (178; 1,448; 991) and active crown decline rating (40, 55, and 80 percent). The proportions of successful colonization based on total numbers of attacks were 92, 53 and 80 percent on the actively declining trees (40, 55, and 80 percent rating, respectively).

Bark cankers and/or xylem lesions were associated with less than 40 percent of the total number of hickory bark beetle attacks (Table 2). The estimate of cankers/lesions is conservative, however, for the tree with 55 percent decline. Evidence of bark beetle attacks were apparent on most 1-m stem sections for this tree, but the cambial region of several sections was uniformly discolored and discrete lesions could not be detected. The mean lengths of the observed xylem lesions on each bark-stripped tree were 8.2, 5.1, and 7.1 cm on the stripped trees (40, 55, and 80 percent crown decline rating, respectively). In all cases, the observed lesions extended beyond the edges of the bark beetle gallery systems when present. *C. smalleyi* is commonly isolated from these types of xylem lesions (Park 2011), but this variable was not assessed for the three trees.

C. *smalleyi* Presence on Hickory Bark Beetles

Only female beetles were captured by probing the entry tunnels that each was constructing during their initial attacks of six bitternut hickories in two northern Wisconsin and one southeastern

Table 1.—Number of adult hickory bark beetle attacks by extent of beetle colonization on three bitternut hickory trees exhibiting active crown decline

Tree number	Location	Tree size		Percent crown decline	Extent of beetle colonization by category		
		d.b.h. (cm)	height (m)		entry tunnel only	tunnel plus egg gallery	full gallery system[a]
1	Chippewa Co., WI	26.7	21.3	80	30	27	934
2	Marathon Co., WI	23.7	21.4	55	401	276	771
3	Marathon Co., WI	22.9	26	40	13	1	164

[a]Full gallery system includes entry tunnel, full egg gallery and radiating larval tunnels.

Table 2.—Numbers of bark cankers and xylem lesions found on main stems of three bitternut hickory trees exhibiting active crown symptoms

Tree number	Location	Tree d.b.h. (cm)	Percent crown decline	Numbers of bark cankers and xylem lesions	
				Total	Associated with beetle attacks
1	Chippewa Co., WI	26.7	80	113	106
2	Marathon Co., WI	23.7	55	585[a]	551
3	Marathon Co., WI	22.9	40	26	24

[a]Cambium of stem sections with numerous hickory bark beetle attacks were dead and uniformly darkened in color; thus, it was not possible to detect xylem lesions with reddish-brown discoloration.

Table 3.—Number of adult hickory bark beetles on which *Ceratocystis smalleyi* was detected via serial dilution plating assay and molecular assay (PCR and cloning). The beetles were collected during their initial attacks on six bitternut hickories between late August and early September 2009.

Collection location	Number of sampled trees	Number of beetles collected	Serial dilution plating results		PCR and cloning results	
			Number of beetles assayed	Number yielding *C. smalleyi*	Number of beetles assayed	Number with *C. smalleyi*
Wabasha Co., MN	1	19	19	2	--[a]	--
Marathon Co., WI	2	154	61	57	12	11
Shawano Co., WI	3	112	60	53	12	11

[a] -- denotes that no beetles were assayed.

Minnesota locations. All collections were made between 26 August and 2 September 2009. *C. smalleyi* was isolated from bark beetles collected from all six trees using serial dilution plating, but isolation frequencies differed by site (Table 3). Isolation rate was lowest for the Minnesota site (11 percent) compared to the Wisconsin sites (88 and 93 percent). Similar results (92 percent pathogen positive) were found using PCR and cloning on washings from a subset (n = 24) of the Wisconsin beetle collections (Table 3). Of 219 clones that were amplified from 24 attacking bark beetles, 93 yielded sequences of *C. smalleyi* (data not shown).

Both male and female beetles (n = 43) were collected just prior to bark beetle emergence by excavation from the bark of two bitternut hickories (19 and 28.7 cm d.b.h.) exhibiting crown decline (50 and 80 percent decline ratings) in the Shawano County, WI, stand. Bark beetles were collected 18-19 June 2010. *C. smalleyi* was isolated from 7 percent of the beetles assayed for the fungus using serial dilution plating.

Table 4.—Proportion of bark area (within the inoculated stem zone) of bitternut hickory exhibiting bark cankers or other necrosis following multiple inoculations with *Ceratocystis smalleyi* prior to sap flow rate measurements

Study location	Treatment [a]	Number of trees	Cankered or necrotic bark area (percent) [b]	
			mean	range
Wabasha Co., MN	*C. smalleyi* inoculated	3	8.8	3.8 - 11.5
	water inoculated	1	0.9	--
	non-inoculated	2	0	--
Chippewa Co., WI	*C. smalleyi* inoculated	5	26.2	15.3 - 41.3
	water inoculated	3	1.3	0.6 - 2.2
	non-inoculated	3	0	--

[a] Aqueous suspensions (0.1 ml) of fungus ascospores were placed in 6 mm dia drilled hole into the outer sapwood; 0.1 ml sterile distilled water was used on water-inoculated trees.

[b] The sum of the areas of the 50 bark cankers (fungus inoculated trees) or necrosis associated with water inoculated wound was divided by the stem surface area in the inoculated height zone to estimate the diseased or necrotic portion of each stem's bark.

Numerous male and female bark beetles emerged from stem sections placed in emergence tubes. The sections were cut from three bitternut hickories (between 23 and 34 cm d.b.h.) exhibiting active crown decline in Shawano County, WI, and four trees (between 14 and 27 cm d.b.h.) exhibiting active crown decline in Marathon County, WI. The declining trees were felled and stem sections collected between 20 May and 19 June 2009. No *C. smalleyi* was isolated from any of the 120 bark beetles assayed using serial dilution plating. The same result was found using PCR and cloning on washings from a subset of the same beetle collections. Of 248 clones that were amplified from 21 emerged adult bark beetles, none yielded sequences of *C. smalleyi* (data not shown).

Effect of Cankers on Tree Sap Flow Rate

Proportion of Inoculated Stems with Bark Cankers

Twelve to fourteen months after *C. smalleyi* inoculation, the sap flow study trees exhibited large bark cankers and accompanying xylem lesions compared to very small necrotic areas on water controls (Table 4). The mean size (area) of the cankers on the Wisconsin trees (64.3 ± 2.2 cm^2) was larger than that on the Minnesota trees (15.3 ± 0.6 cm^2) ($P < 0.0001$). The sum of the areas of the 50 bark cankers on each tree was calculated and divided by the stem surface area in the inoculated zone to estimate the proportion of each diseased stem. The proportions calculated for the fungus-inoculated trees were variable (up to 11.5 percent in Minnesota and 41.3 in Wisconsin), but clearly much larger than necrotic bark areas on water-inoculated control trees (< 2.2 percent) (Table 4).

Diurnal Patterns of Sap Flow Rates

Similar diurnal trends of sap flow were found for all the trees (Fig. 1). Sap flow rate was highest just after 12 noon and lowest through the nighttime. Lower sap flow rates during midday were found for the *C. smalleyi* inoculated trees compared to the water inoculated controls.

The maximum values of sap flow rate on a representative number of days within the measurement period were lowest for the fungus-inoculated trees (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) compared to the control trees (26.3 to 30.0 g m^{-2} s^{-1}, Minnesota; 34.2 to

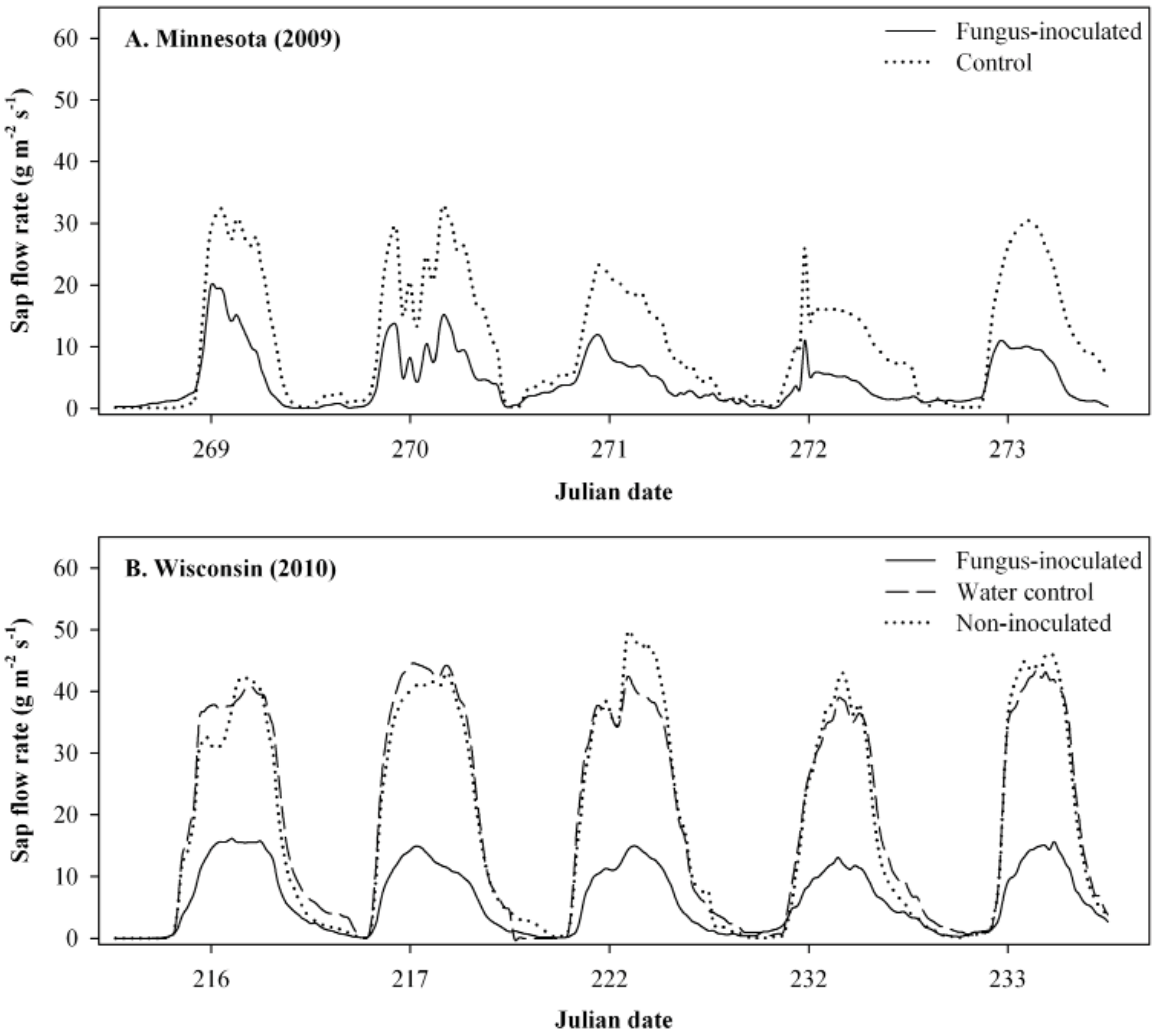

Figure 1.—Diurnal patterns of sap flow rates in bitternut hickory trees (13 to 28 cm d.b.h.) inoculated with *Ceratocystis smalleyi*. Measurements displayed are a subset of the longer time period during which sap flow was measured and recorded.

49.3 g m^{-2} s^{-1}, Wisconsin) (Fig. 1). In Minnesota, the average maximum sap flow rate of all infected trees (14.0 ± 2.1 g m^{-2} s^{-1}) was 51 percent lower than the average maximum value for the control trees (28.6 ± 1.1 g m^{-2} s^{-1}) (P = 0.009). In Wisconsin, the average maximum sap flow rate of all infected trees (15.3 ± 3.8 g m^{-2} s^{-1}) was 64 percent lower than the mean maximum value for all the control trees (41.9 ± 2.2 g m^{-2} s^{-1}) (P=0.0001). The sap flow rates were consistently lower in the Minnesota trial than the Wisconsin trial, but this was likely due to the differences in time of year during which measurements were made, i.e., early fall in Minnesota versus midsummer in Wisconsin.

Tylose Abundance, Cankered Stem Area and Sap Flow Rate Relationships

Differences in vessel diameters between inoculated trees and control trees were insignificant (P ≥ 0.19) (data not shown). Vessel diameter distributions of the inoculated trees and the controls were also similar (P = 0.23). Tyloses were observed within vessels of all the trees, but they were more abundant in the *C. smalleyi* inoculated ones than the controls (P < 0.006) in both the two outer annual rings (9 percent, controls; 30 to 56 percent, fungus-inoculated) and the outer 9 to 10 outer annual rings (25 to 42 percent, controls; 37 to 59 percent, fungus-inoculated). The difference in

Table 5. —Mean length and width of inner bark lesions and mean length of discolored sapwood on *Ceratocystis smalleyi* inoculated bitternut hickory 2 and 12 months after treatment

Time after inoculation (months)	Inoculum	Number of trees	Total number of inoculation points	Mean inner bark lesion dimension (cm)		Discolored sapwood
				width	length	length (cm)
				+/- SE	+/- SE	+/- SE
2	*C. smalleyi* [a]	4	16	2.6 ± 0.08	21.8 ± 2.64	23.6 ± 2.84
2	sterile water	2	8	0.2 ± 0.02	0.2 ± 0.02	0
12	*C. smalleyi*	2	8	3.0 ± 0.09	64.2 ± 3.20	68.0 ± 3.27

[a] Data were pooled from inoculated trees with two *C. smalleyi* isolates (CS0731 and CS0734).

tylose abundance between treatments was more pronounced in the outer two annual rings ($P = 0.0012$, Minnesota; $P < 0.0001$, Wisconsin). Tyloses rarely formed in the most recent xylem vessels in the absence of fungal infection. *C. smalleyi* inoculated trees had more vessels with tyloses in the outer 9 to 10 annual rings than the controls ($P < 0.006$) for both sites.

Correlation analyses revealed significant interactions between 1) average maximum sap flow rate and tylose abundance in the two outer annual rings ($P = 0.0084$) for both sites combined, and 2) tylose abundance in the same anatomical location and proportion of cankered bark ($P = 0.0045$) for both sites combined. Correlation analysis also revealed significant interactions between average maximum sap flow rate and proportion of cankered bark area ($P = 0.0042$). Specifically, an inverse relationship was found between percent of proportion of cankered bark area and average maximum sap flow rate in both sites based on linear regression analysis (Minnesota: $R^2 = 0.90$, $P = 0.0042$; Wisconsin: $R^2 = 0.90$, $P < 0.0001$).

Bitternut Hickory Stem Colonization by *Ceratocystis smalleyi*

Bark Canker Development

Long narrow cankers were evident two months after inoculation with *C. smalleyi* isolates while no cankers resulted from water (control) inoculation. Mean dimensions of inner bark cankers (length and width) were greater at 12 months than 2 months ($P < 0.05$) based on two-sample t-tests for means of measurements at each inoculation point (Table 5). Long narrow, reddish-brown discoloration of sapwood was associated with each fungus inoculated point. Wedge-shaped discoloration was evident in cross-sections of the stem samples. Wound callus formed over all water-inoculated points and no sapwood discoloration was found. In general, the length of the sapwood discoloration was larger than the corresponding inner bark lesion.

Visible Evidence of Fungus Infection in Sapwood

C. smalleyi was isolated from the margins of discolored and adjacent clear sapwood, i.e., the reaction zone or canker margin, and never isolated from sapwood associated with water-inoculation points. Using light microscopy and toluidine blue O stain, fungal hyphae were commonly observed and found to be abundant in the discolored sapwood at the canker margins. Hyphae were commonly observed in the different elements of the xylem tissue, e.g., axial and radial parenchyma cells, fibers, and vessels (Fig. 2 A and B). Furthermore, hyphae were found to grow through bordered

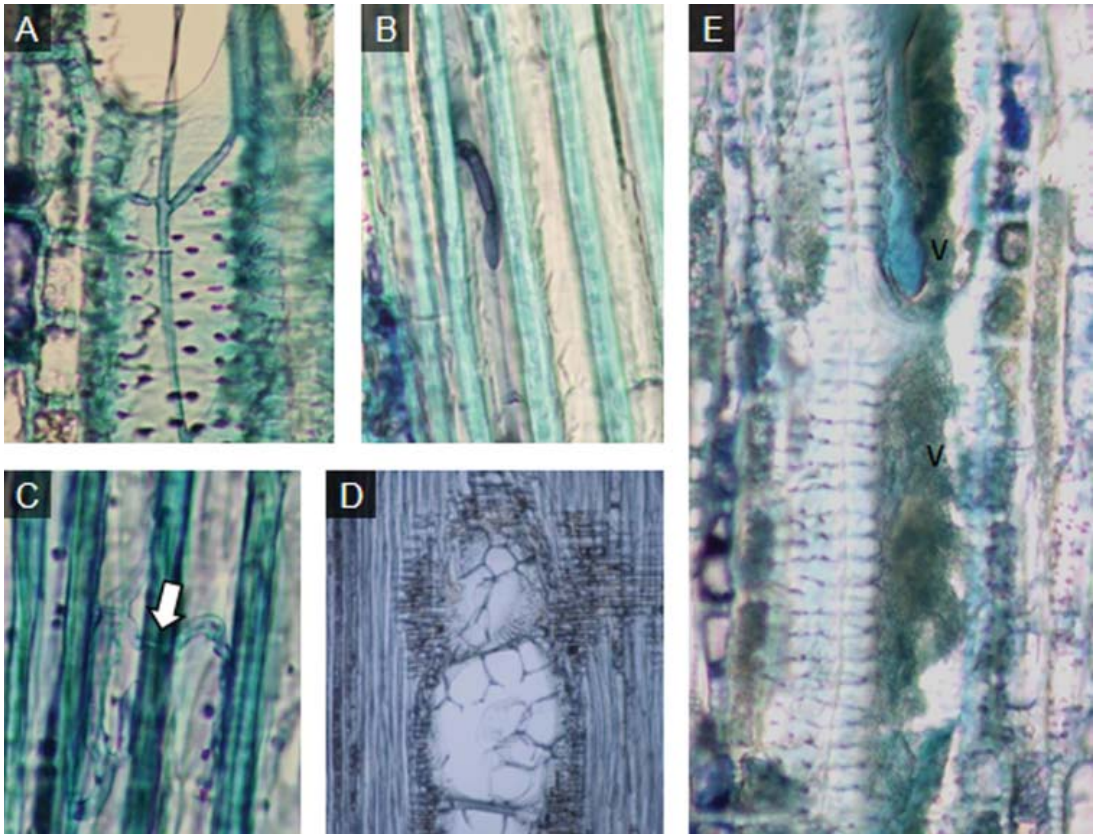

Figure 2.—Presence of *Ceratocystis smalleyi* and host responses to its colonization in sapwood of bitternut hickory. A. Branching of hyphae in a large vessel. B. Advancing hyphal tip in a wood fiber. C. Hyphal penetration of a bordered pit. D. Earlywood vessel showing contiguous tyloses in radial view. E. Gels formed in latewood vessel (V) and surrounding parenchyma cells in radial view. Sections A, B, C, and E were stained with toluidine blue O and section D was stained with Nile blue.

pits between neighboring cells (Fig. 2 C). No fungal hyphae were observed in sapwood of water inoculated trees. Fungal hyphae were observed in 0.4 to 12.4 percent of nonoccluded vessels in the reaction zone at 2 months and 0.4 to 34.7 percent of vessels at 12 months after treatment in fungus-inoculated trees. The mean proportion of fungus-colonized vessels was higher at 12 months (8.1 ± 2.1 percent) than at 2 months (2.1 ± 0.7 percent) ($P = 0.01$).

Xylem Occlusion by Tyloses and Gels

Tyloses were abundant in early-wood vessels of the fungus-inoculated trees and vessel lumens typically occluded by multiple, contiguous tyloses (Fig. 2 D). Gels partially or completely occluded late-wood vessels. When vessels were completely occluded, gel substances were observed commonly in parenchyma cells surrounding the affected vessel(s) (Fig. 2 E). Vessel occlusion occurred in response to both water and fungus inoculation, but was more abundant ($P < 0.05$) in *C. smalleyi* inoculated trees. No gel accumulation was observed in water-inoculated trees.

DISCUSSION

Nature of Hickory Bark Beetle–*C. smalleyi* Interaction

Based on the literature, death of hickory attributed to hickory bark beetles is caused by girdling of the main stem of the affected tree by coalescing galleries of the bark beetle (U.S. Forest Service 1985). Our observation, however, is that hickory bark beetle gallery systems do not coalesce on actively declining trees, and when *C. smalleyi* xylem lesions are associated with the galleries, the lesion areas in the cambium almost always exceeds the area occupied by the gallery system. Coalescing beetle galleries were observed on dead standing bitternut hickory during the 2007-2008 hickory survey (Juzwik et al. 2008b), but we suspect such extensive colonization of the bark occurs after 95 percent or more of the crown has died.

Based on results of studies presented in this paper and unpublished field observations, we propose that a synergistic interaction of hickory bark beetles and *C. smalleyi* is likely responsible for the observed rapid crown decline and subsequent death of affected, smooth-bark hickories. *C. smalleyi* was detected on a high percentage of attacking beetles collected from two of three field sites. In addition, reddish-brown inner bark and xylem lesions were often observed in mid-September on recently attacked trees (J. Juzwik, pers. observation). The adult bark beetle thus provides the entry and infection court for the fungus on susceptible hickories. Furthermore, the bark beetle may be responsible for dissemination and inoculation of the fungus into the bark beetle wounded tissues. The fruiting bodies (perithecia) of the homothallic fungus have been observed in galleries of the hickory bark beetle and the sticky spores produced by the perithecia are well-suited for acquisition by emerging beetles (Johnson et al. 2005). However, in this study dilution plating and PCR and cloning indicated that none of the assayed bark beetles that emerged from stems of heavily attacked, actively declining bitternut hickory yielded the fungus. Several explanations for this lack of pathogen detection include: the numbers of bark beetles assayed were too few to detect the fungus in the sampled population of beetles emerging from the trees, the beetle is not an important disseminator of the fungus, or the beetle acquires the fungus from another source following its early summer emergence from beetle-killed trees but prior to attack of other hickory in late summer. Additional study is needed to address these hypotheses and to determine if the insect is an important vector of the pathogen or merely provides an infection court.

Role of *C. smalleyi* in Rapid Crown Decline

Histological investigations associated with the sap flow study and the fungus colonization study demonstrated *C. smalleyi* infections in sapwood of bitternut hickory apparently induce tylose formation in xylem vessels and accumulation of gels in late-wood vessels and surrounding parenchyma cells. Both of these response mechanisms lead to occlusion of xylem vessels and obstruction of water transport in affected trees. Reduced sap flow rates in bitternut hickory with multiple stem infections apparently result from these obstructions. Multiple fungus infections and resulting host response can logically explain the symptom of rapid crown decline (and at times, foliage wilt) observed in affected trees.

Growth and spread of the fungus through xylem vessels may be responsible for canker resurgence observed on both naturally infected and artificially inoculated trees. This resurgence may account for the occurrence of xylem lesions and bark cankers that infrequently occur in the absence of hickory bark beetle attack on stems of affected bitternut hickory. Conspicuous lack of fungus spores in the xylem supports the observation that systemic spread of the pathogen throughout the tree

(e.g., as occurs with *Ceratocystis fagacearum* in red oaks in development of oak wilt) does not occur. Furthermore, the long but restricted xylem lesions associated with *C. smalleyi* infection and the host response to infection (e.g., tylose occluded vessels) supports the hypothesis that the fungus is a limited vascular wilt pathogen in bitternut hickory. This situation is similar to that of *Raffaelea quercivora* infections of *Quercus crispula* and *Q. serrata* following ambrosia beetle attacks in the disease known as Japanese oak wilt (Kuroda 2001, Kuroda and Yamada 1996, Murata et al. 2005, Murata et al. 2007) and to *Phytophthora ramorum* infection of sapwood and host response in tanoak (Collins et al. 2009, Parke et al. 2007).

Hickory Decline as the Disease Name

Recent reports of dieback or of declining crowns of bitternut and shagbark hickory exist in the literature (Juzwik et al. 2008a, Wisconsin DNR 2005). The specific disease of rapidly declining crowns and mortality in smooth-bark hickory is an example of a previously described decline disease for which major determinants are now known (Ostry et al. 2011). Through sequential and systematic studies, we have demonstrated that mass attacks by hickory bark beetles and closely associated xylem lesions caused by the canker fungus, *C. smalleyi*, are the most common stem damage associated with actively declining bitternut hickory. With this new knowledge, we propose that the new name "Ceratocystis wilt of hickory" be formally used for this disease. This follows the history of a newly discovered disease of black walnut that was first described as walnut decline in the 1990s, but upon discovery of the primary biotic causes (walnut twig beetle and *Geosmithia morbida*) the name was changed to better describe the disease, i.e., thousand cankers disease (Tisserat et al. 2009).

In conclusion, declining crowns and death of hickory have been previously attributed to drought and subsequent hickory bark beetle attacks (U.S. Forest Service 1985) or to a decline disease of the species (Juzwik et al. 2008a, Wisconsin DNR 2005). Results from the series of related studies presented in this paper document the deleterious effects of multiple cankers and xylem dysfunction caused by *C. smalleyi* on the health of bitternut hickory. Furthermore, the synergistic interaction of the hickory bark beetle and *C. smalleyi* results in numerous bark cankers and debilitating xylem lesions on stems of bitternut hickory that we hypothesize leads to rapid crown decline and tree death, especially following predisposing abiotic events. Drought is still considered to be an important predisposing factor as it leads to hickory bark beetle population buildup.

ACKNOWLEDGMENTS

This work was partially funded by U.S. Forest Service, Forest Health Evaluation Monitoring Grant FHEM- NC-EM-07-01. Additional funding (salary, benefits, and tuition) for the second author was provided by the University of Minnesota, Department of Plant Pathology. The authors thank J. Hickey (private landowner), B. Stempa (US Bureau of Indian Affairs) and the Stockbridge-Munsee Community, D. Richardson and the Chippewa County Forest, and the Minnesota Department of Natural Resources (Carley State Park) for providing access to lands and trees for these studies. The authors gratefully acknowledge the field research coordination by P. Castillo and the technical assistance of M. Boll, P. Castillo, J. Hickey, A. Klein, B. Stempa, K. Sweeny, B. Davids, and B. Zenner. The sap flow studies would not have been possible without the guidance of E. Peters, J. Shannon, and J. Cavender-Bares. The training in wood microtomy provided by A. Wiedenhoeft was invaluable for the histological studies.

LITERATURE CITED

Collins, B.R.; Parke, J.L.; Lachenbruch, B.; Hansen, E.M. 2009. **The effects of *Phytophthora ramorum* infection on hydraulic conductivity and tylosis formation in tanoak sapwood.** Canadian Journal of Forest Research. 39: 1766-1776.

Goeden, R.D.; Norris, D.M., Jr. 1964. **Some biological and ecological aspects of the dispersal flight of *Scolytus quadrispinosus* (Coleoptera: Scolytidae).** Annals of the Entomological Society of America. 57: 743-749.

Granier, A. 1987. **Evaluation of transpiration in a Douglas-fir stand by means of sap flow measurements.** Tree Physiology. 3: 309-320.

Hopkins, A.D. 1912. **The dying hickory trees—cause and remedy.** Circ. 144. Washington, DC: U.S. Department of Entomology, Bureau of Entomology. 5 p.

Johnson, J.A.; Harrington, T.C.; Engelbrecht, C.J.B. 2005. **Phylogeny and taxonomy of the North American clade of the *Ceratocystis fimbriata* complex.** Mycologia. 97: 1067-1092.

Juzwik, J.; Haugen, L.; Park, J-H.; Moore, M. 2008a. **Fungi associated with stem cankers and coincidental scolytid beetles on declining hickory in the Upper Midwest.** In: Jacobs, D.F.; Michler, C.H., eds. Proceedings, 16th central hardwoods forest conference. Gen. Tech. Rep. NRS-P-24. Newtown Square, PA: U.S. Department of Agriculture, Forest Service, Northern Research Station: 476-482.

Juzwik, J., Olszewski, S., Park, J-H., Reynolds, G.; Haugen, L. 2008b. **Assessment and etiology of hickory decline–preliminary results [Poster]**. In: 2008 annual U.S. Forest Service Forest Health Monitoring working group poster presentation; Feb 11-14; Ron Billings, TX. Available at www.fhm.fs.fed.us/posters/posters08/posters08.shtm. (Accessed July 5, 2011).

Juzwik, J., Castillo, P., Park, J-H., and Haugen, L. 2009. **Assessment and etiology of hickory decline: Second year results** [Poster]. In: 2009 annual U.S. Forest Service Forest Health Monitoring working group poster presentation; February 23-27; Savanah, GA. Available at www.fhm.fs.fed.us/posters/posters09/posters09.shtm. (Accessed July 5, 2011).

Kuroda, K. 2001. **Responses of *Quercus* sapwood to infection with the pathogenic fungus of a new wilt disease vectored by the ambrosia beetle *Platypus quercivorus*.** Journal of Wood Science. 47: 425-429.

Kuroda, K.; Yamada, T. 1996. **Discoloration of sapwood and blockage of xylem sap ascent in the trunks of wilting *Quercus* spp. following attack by *Platypus quercivorus*.** Journal of Japanese Forest Society. 78: 84-88. (in Japanese with English summary)

Lindner, D.L.; Banik, M.T. 2009. **Effects of cloning and root-tip size on observations of fungal ITS sequences from *Picea glauca* roots**. Mycologia. 101: 157-165.

Lu, P.; Urban, L.; Ping, Z. 2004. **Granier's thermal dissipation probe (TDP) method for measuring sap flow in trees: theory and practice**. Acta Botanica Sinica. 46: 631-646.

Murata, M.; Yamada, T.; Ito, S. 2005. **Changes in water status in seedlings of six species in the Fagaceae after inoculation with *Raffalelea quercivora* Kubono et Shin-Ito**. Journal of Forest Research – Japan. 10: 251-255.

Murata, M.; Yamada, T.; Matsuda, Y.; Ito, S. 2007. **Discoloured and non-conductive sapwood among six Fagaceae species inoculated with *Raffaelea quercivora*.** Forest Pathology. 37: 73-79.

New York State Museum. 1910**. 25th report of the state entomologist on injurious and other insects of the State of New York 1909**. In: Education Department Bulletin No. 475, Museum Bulletin 141. Albany, NY: University of the State of New York: 103-104.

Ostry, M.E.; Venette, R.C.; Juzwik, J. 2011. **Decline as a disease category: Is it helpful?** Phytopathology. 101: 404-409.

Park, J-H. 2011. **Etiology of crown decline and dieback in bitternut hickory**. St. Paul, MN: University of Minnesota. 122 p. Ph.D. dissertation.

Park, J.-H.; Juzwik, J.; Haugen, L.M. 2010. **Ceratocystis canker of bitternut hickory caused by *Ceratocystis smalleyi* in the North-Central and Northeastern United States.** Plant Disease. 94: 277.

Parke, J.L.; Oh, E.; Voelker, S.; Hansen, E.M.; Buckles, G.; Lachenbruch, B. 2007. ***Phytophthora ramorum* colonizes tanoak xylem and is associated with reduced stem water transport.** Phytopathology. 97: 1558-1567.

Peters, E.B.; McFadden, J.P.; Montgomery, R.A. 2010. **Biological and environmental controls on tree transpiration in a suburban landscape.** Journal of Geophysical Research. 115, G04006. DOI: 10.1029/2009JG001266.

Smith, D.M.; Allen, S.J. 1996. **Measurement of sap flow in plant stems.** Journal of Experimental Botany. 47: 1833-1844.

Solomon, J.D.; Payne, J.A. 1986. **A guide to the insect borers, pruners, and girdlers of pecan and hickory.** Gen. Tech. Rep. SO-64. New Orleans, LA: U.S. Department of Agriculture, Forest Service, Southern Forest Experiment Station. 31 p.

Steinman, J. 2004. **Forest health monitoring in the northeastern United States: disturbances and conditions during 1993-2002.** NA-TP-01-04. Newtown Square, PA: U.S. Department of Agriculture, Forest Service, Northeastern Area State and Private Forestry. 46 p. Available at http://fhm.fs.fed.us/pubs/tp/dist_cond/hardwoods/hickory.pdf. (Accessed July 5, 2011).

Tisserat, N.; Cranshaw, W.; Leatherman, D.; Utley, C.; Alexander, K. 2009. **Black walnut mortality in Colorado caused by the walnut twig beetle and thousand cankers disease.** Plant Health Progress. DOI: 10.1094/PHP-2009-0811-01-RS.

U.S. Forest Service. 1985. **Insects of eastern forests**. Misc. Pub. No. 1426. Washington, DC: U.S. Department of Agriculture, Forest Service. 608 p.

U.S. Forest Service. 2011. **National forest health monitoring.** Arlington, VA: U.S. Department of Agriculture, Forest Service, National Forest Health Monitoring. Available at http://www.fhm.fs.fed.us/. (Accessed July 5, 2011).

U.S. Forest Service. 1994. **Pest alert: hickory mortality.** NA-PR-02-94. Radnor, PA: U.S. Department of Agriculture, Forest Service, Northeastern Area State and Private Forestry. 2 p.

Wisconsin Department of Natural Resources. 2005. **Wisconsin forest health highlights, December 2005.** Available at http://fhm.fs.fed.us/fhh/fhh-05/wi/wi_05.pdf. (Accessed July, 2011).

The content of this paper reflects the views of the authors(s), who are responsible for the facts and accuracy of the information presented herein.