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Ceratocystis smalleyi colonization of bitternut hickory and host responses in the xylem

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Summary

Colonization of *Carya cordiformis* sapwood by *Ceratocystis smalleyi* and subsequent host defence responses following artificial inoculation were investigated using anatomical and histological techniques. Hyphae of *C. smalleyi* were observed in all sapwood xylem features confirming the ability of the pathogen to invade and colonize the xylem tissues of the host species. The fungus was isolated from within and at the margins of discoloured sapwood areas at 2 and 12 months after inoculation. General host defence responses that included vessel occlusion with gels or tyloses, lipid accumulation, and production of phenolic compounds were observed in xylem tissues of inoculated *C. cordiformis* stems. Pectic substances, lipids, and to a rare extent, phenolic compounds were detected in vascular gels. The lipid-rich barriers observed likely prevent lateral expansion of the fungus in the sapwood. Furthermore, lack of fungus sporulation within vessels may restrict axial spread of the fungus. *C. smalleyi* appears to be a limited vascular wilt pathogen of bitternut hickory based on these observations and previously reported sap flow reduction correlated with multiple infections in artificially inoculated trees.

1 Introduction

Severe decline and mortality of hickory (*Carya* spp.) has occurred periodically in the eastern United States since the early 1900s (US Department of Agriculture, Forest Service 1985; Steinman 2004). A 2007–2008 field survey of declining stands in six states found three major types of symptomology: (i) top dieback, (ii) slow crown decline and (iii) rapid crown decline (Juzwik et al. in press). This situation is currently viewed as a complex of at least three diseases and all are considered to contribute to the observed decline and mortality of hickory (Juzwik et al. in press; Ostry et al. 2011). Top dieback was commonly attributed to numerous Fusarium cankers on the main stem. Numerous branch galls and/or stem-encircling galls attributed to *Phomopsis* sp. was the disease commonly associated with observed slow crown decline. The major disease on bitternut hickory (*Carya cordiformis*), however, is the one characterized by rapid crown decline and subsequent tree death. A synergistic interaction of heavy attack by the hickory bark beetle (*Scolytus quadrispinosus*) and development of numerous Ceratocystis cankers commonly associated with the attacks on stressed hickory is hypothesized as the cause of this disease.

The hickory bark beetle historically has been associated with widespread mortality of hickory (US Department of Agriculture, Forest Service 1985). In the late 1980s, a *Ceratocystis* species was detected on hickory-bark-beetle-attacked trees (US Department of Agriculture, Forest Service, Northern Area State and Private Forestry 1994). The fungus species was described and named as *Ceratocystis smalleyi* in 2005 (Johnson et al. 2005). Our investigation of the role of *C. smalleyi* in bitternut hickory decline began in 2006.

C. smalleyi was first reported as a bark canker pathogen on poletimber- and sawtimber-size bitternut hickory in 2010 (Park et al. 2010). Reddish-brown discoloration or stain of the sapwood also was consistently associated with fungus-inoculated stems. Wedge-shaped, reddish-brown discoloration extending from the cambium to the sapwood–heartwood boundary is observed commonly in cross sections of cut stems of naturally infected trees (Juzwik and Park, personal observations). Thus, investigation of the colonization of sapwood by *C. smalleyi* and the resulting impact on tree health was initiated. Field observations, pathogenicity tests and a physiological study provided evidence for its ability to function as a limited vascular wilt pathogen rather than an innocuous stain fungus (Juzwik et al. in press; Johnson et al. 2005; Park et al. 2013).

Discoloration in sapwood (or young xylem) is considered a diagnostic trait of xylem pathogens (Sinclair and Lyon 2005). Patterns of xylem discoloration caused by *C. smalleyi* (e.g. wedge-shaped, local lesion, radial and vertical expansion) are different from vascular streaking in the outer sapwood caused by true vascular pathogens such as *Ophiostoma ulmi, C. fagacearum, Fusarium oxysporum* f.sp. *perniciosum* and *Verticillium dahliae* (Phipps and Stipes 1976; Sinclair and Lyon 2005). Patterns similar to *C. smalleyi* in hickory have been documented for canker stain of plane trees, sudden oak death of tan oak, Japanese oak wilt and Phytophthora stem diseases of hardwood trees (Kuroda and Yamada 1996; Panconesi 1999; Brown and Brasier 2007; Parke et al. 2007). Although none of these diseases are systemic wilts, the pathogen of each of the diseases was found or suggested to be able to establish itself in xylem tissues. Likewise, it is likely that the xylem discoloration of diseased bitternut hickory is a result of sapwood colonization by *C. smalleyi*. High frequency of isolation of *C. smalleyi* from the discoloured sapwood supports this speculation (Juzwik et al. in press).

Another very interesting feature is the occurrence of bark lesions that arise from discoloured sapwood and speculated vertical spread of the pathogen through xylem elements (Park et al. 2013). Multiple phloem lesions associated with a vertically expanding strip of discoloured xylem are referred to as secondary lesions, canker resurgences, necrotic reappearances,

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or island lesions (Panconesi 1999; Brown and Brasier 2007). It is possible that secondary infection occurs from an outside source such as natural infections initiated by pathogen propagules disseminated via wind-driven rain, insect vectors or other means. However, secondary phloem lesions produced during pathogenicity tests (Park et al. 2010) provide evidence for the ability of the pathogen to spread through vessels and break out from the xylem into unaffected phloem tissues, thus initiating a new phloem lesion.

Young xylem plays a vital role in tree physiology by transporting water and minerals through vessels from roots to crowns and storing food in living parenchyma cells (Pallardy 2008). Due to the crucial role of the xylem in conducting water, hydraulic disruption caused by xylem infection of a pathogen has been examined for several stem canker and stain diseases as well as for true vascular wilt pathogens (Beckman 1987; Yamaoka et al. 1990; Kuroda and Yamada 1996; Kirisits and Offenthaler 2002; Murata et al. 2005; Parke et al. 2007; Collins et al. 2009). In most cases, xylem vessels lose their functionality when colonized with propagules of a pathogen or due to embolism or vessel occlusion resulting from host defence responses. Such dysfunction of water conduction was observed in bitternut hickory infected with *C. smalleyi* where reduced sap flow was closely associated with disease severity when defined as extent of bark necrosis (Park et al. 2013). On this basis, we hypothesized that xylem infection by *C. smalleyi* and accompanying tree defence responses in the xylem contribute to rapid crown decline in affected trees.

As a xylem pathogen infects living sapwood, a host tree responds with a range of defence mechanisms. The induced host reactions include cell wall alterations such as lignification and suberization of parenchyma cell walls, production of constitutive and induced antimicrobial compounds and occlusion of xylem elements with tyloses or gels (Beckman 1987; Pearce 1996; Yamada 2001). In bitternut hickory infected with *C. smalleyi*, tyloses were frequently formed in xylem vessels (Park et al. 2013). The extent of vessel occlusion by tyloses was correlated with disease severity as well as loss of water conduction in *C. smalleyi*-infected trees (Park et al. 2013). Although tylose formation is a part of the natural process of xylem senescence from sapwood to heartwood, it mostly occurs in vessels of young xylem of many hardwood species as a result of wounding, or infection from fungi or bacteria (Tyree and Sperry 1989; Kuroda 1991; Sun et al. 2007). We hypothesize that the tylose proliferation observed in *C. smalleyi*-infected bitternut hickory occurs as a part of the sapwood defence response to xylem infection because wounding of control trees did not induce a comparable abundance of tyloses (Park et al. 2013). It is possible that other defence responses might have occurred in the sapwood following *C. smalleyi* infection in the same physiological study.

Success of a pathogen in establishing itself in host tissues depends on how rapidly and intensely the host defence responses act to contain the pathogen in the initial infection site (Dimond 1970). Tree species that are highly susceptible to *C. fagacearum* have slow initial responses that would otherwise limit systemic spread of the pathogen (Jacobi and MacDonald 1980). Some wilt pathogens such as *O. ulmi* produce cell wall degrading enzymes, toxins and growth substances that also help overcome defence responses (Dimond 1970; Beckman 1987; Brasier 1991). Based on results in the physiological study (Park et al. 2013), *C. smalleyi* invasion of hickory xylem appears to be limited to the discoloured zones with some vertical movement in the vascular system. Thus, *C. smalleyi* does not seem to behave as a true vascular wilt pathogen. The reaction zone associated with the xylem lesion margin would be an appropriate area for an investigation of the host–pathogen interaction. The reaction zone is the portion of the xylem that is physically and chemically modified in response to fungal invasion and where host defence responses actively occur (Pearce 1991).

Histological and anatomical investigations were undertaken to further explore the hypothesis that sapwood infection by *C. smalleyi* and the accompanying tree defence responses likely contribute to rapid crown decline in bitternut hickory by causing vascular system dysfunction. Of particular interest was the manner in which *C. smalleyi* (i) colonizes sapwood of bitternut hickory, ii) induces defence responses in infected trees and (iii) affects an infected tree's hydraulic function. The specific objectives of the study reported here were: (i) to document the colonization of sapwood by *C. smalleyi* spatially and temporally following artificial inoculation and (ii) to identify host defence responses against the pathogen using anatomical and histological approaches and light microscopy.

2 Materials and methods

2.1 Study site and trees

The study site was located in a mixed hardwood stand in Chippewa Co., WI (45°11′ 5″N; 91°21′ 26″W). Bitternut hickory comprised approximately 35% of the trees in the stands. Eighteen poletimber-sized bitternut hickory trees (13–28 cm in diameter at 1.4 m) with healthy crowns (<15% visible dieback) and stems without visible defect or damage were selected for inoculations. The trees were intermediate to dominant in the canopy.

2.2 Fungus inoculation

Two isolates of *Ceratocystis smalleyi* (CS0731 and CS0734) were selected for inoculation. Both isolates were obtained during 2007 from bitternut hickory with diffuse cankers and/or hickory bark beetle galleries in Monroe Co., WI. To prepare fungal inoculum, ascospores of *C. smalleyi* were collected from extruded masses on tips of perithecia of 1–2-week-old cultures on 2% malt yeast extract agar (MYEA) and suspended in 1.0 ml 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 1×10^4 ascospores/ml. In June 2008, four holes (0.6 cm diameter) were made by drilling with a sterilized drill bit through the bark surface (sprayed with 70% EtOH) just into the outer sapwood (<1 cm) at 1.2 m stem height on the main stem of

eighteen trees. The four holes were evenly distributed along the tree's circumference. Aliquots (0.1 ml) of the fungal inoculum (1.0×10^4 ascospores/ml) and sterile distilled water were pipetted into the four drilled holes on each of four trees, respectively. Holes were covered with cotton moistened with sterile distilled water and secured with masking tape.

2.3 Temporal sampling and sample processing

Trees were monitored after 2 or 12 months for any crown symptoms such as yellowing, crown thinning and branch dieback. Four trees inoculated with *C. smalleyi* and two trees inoculated with water were harvested 2 months after treatment. Only two, fungus-inoculated trees were harvested 12 months after treatment because the remaining study trees were inadvertently removed by forestry crews during a clear-cut and salvage operation in the stand during winter 2008–2009. The stem section (1.0 m in length) of each tree that included four fungus or water inoculations was cut and transported to the laboratory. Each section was visually examined in the laboratory for any symptoms of diffuse bark cankers and xylem lesions or discoloration. The outer bark of each stem section was removed with a drawknife to reveal inner bark lesions. Width and length of inner bark necrosis associated with each inoculation hole were measured and recorded. The remaining bark was removed to expose discoloration in the outer sapwood that was associated with bark necrosis. Only the length of each xylem lesion was measured because xylem lesions consistently extended radially inward to the outer margin of the heartwood for all fungus-inoculated points. Following bark peeling, each stem section was quartered using a band saw to separate each canker or control wound. For fungus recovery, 4-cm-thick (= height) wood cross sections were taken from the top and bottom of each canker and from each inoculation wound in the case of water inoculation (Fig. 1). For the histological study, three 2-cm-thick wood cross sections per inoculation area were taken from the stem quarter at three points (Fig. 1). Points 1, 2 and 3 were, respectively, 2 cm, 12 cm and 22 cm above each inoculation point. When the wood sections contained discoloured sapwood, two samples (wood cubes of $1.5 \times 1.5 \times 2$ cm) were excised from the outer sapwood. These included the outer 10 annual rings at both lateral margins of the observed discoloration. Thus, each sample consisted of half discoloured tissues and half unaffected tissues (i.e. the presumed reaction zone). The outermost 10 annual rings were selected for observation because they represent most of the functional sapwood area of a ring-porous tree. This location was based on published studies of water transport in large diameter oak trees that are ring-porous, similar to bitternut hickory. In oaks, the highest volume of water (80%) was transported in the outer 1.1 cm of sapwood (Granier et al. 1994). Furthermore, sap flow velocity in oaks was found to decrease sharply (from 50 to 5%) based on measurements taken from the outer three to the ninth growth ring (Čermák et al. 1992). The samples were fixed in FAA solution (formaldehyde: acetic acid: 50% ethanol = 5 : 5 : 90 v/v) prior to sectioning.

Reisolation of *C. smalleyi* was attempted with the stem cross sections obtained from the top and bottom of each canker or from each inoculation wound (for water control) for both 2- and 12-month samples. The stem sections were cut into small wood cubes using a mitre cutter and placed in moist chambers (100-mm-diameter glass petri dishes with moist filter paper circles) maintained at room temperature (approximately 25°C) to stimulate fungus sporulation. The wood cubes were examined under a stereomicroscope at 10 days for the presence of any fungal fruiting bodies. When present, ascospore masses on tips of perithecia sporulating on the wood cubes were transferred to 2% MYEA amended with 100 ppm streptomycin sulphate per litre. Otherwise, wood cubes were kept in the same moist chamber for 10 more days and then re-examined.

Fixed samples for histology were thoroughly washed in running tap water. Of two samples obtained at each sampling point, one sample was sectioned transversely and the other longitudinally to a 20–25 μm thickness using a sliding microtome (Model 860, American Optical, Southbridge, MA, USA). The sections were stained with 0.1% toluidine blue O as a general stain (Clark 1981), 0.1% Nile blue to detect lipid (Chayen and Bitensky 1991), 0.005% ruthenium red for pectin detection (Jensen 1962), 0.05% 4-nitrosophenol in concentrated H_2SO_4 for phenolic compounds (Gersbach et al. 2001) and phloroglucinol (1% aq.) plus HCl for lignin (Redman et al. 1999). Stained sections were subsequently mounted in 10% glycerol on glass slides and viewed with a microscope (Nippon Kogaku Inc., Garden City, NY, USA).

2.4 Data analysis and xylem tissue evaluation

Effects of inoculum type (water *vs.* fungus) and time as inoculation on symptom development (the size of inner bark lesions and sapwood discoloration) were analysed by multivariate Analysis of Variance (MANOVA). As there was no difference

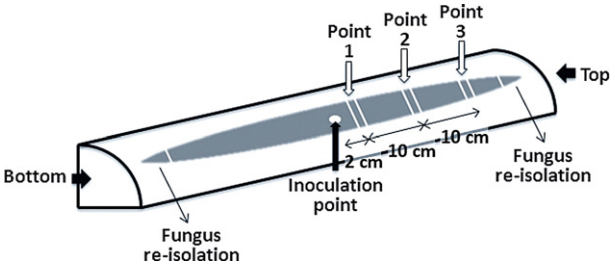

Fig. 1. Sampling scheme for a quarter-sawn stem section of *Ceratocystis smalleyi*-inoculated bitternut hickory. Samples for attempted fungus recovery were taken from upper and lower margins of a canker. Samples for histology were taken at point 1, 2 and 3.

in pathogenicity of two fungal isolates (CS0731 and CS0734) in a previous study (Park et al. 2010), data from the two inoculum types were pooled. Differences of means were determined using Tukey's HSD at the significance level of $\alpha = 0.05$. Normality of data was checked by numerical methods (the Kolmogrov-Smirnov test, Cramer-von Mises test and Anderson-Darling test in the Univariate Procedure in SAS). All statistical analyses were conducted using SAS 9.1 (SAS Institute, Inc. Cary, NC, USA).

The presence of fungal structures, tyloses and gels in vessels were observed in transverse sections stained with toluidine blue O. For each section, 250 vessels were examined with the light microscope to count the number of: (i) occluded vessels with tyloses or gels, (ii) non-occluded, fungus-colonized vessels and (iii) unaffected vessels. Vessels without fungal hyphae, gels and tyloses were designated unaffected. Occurrences of the above features were recorded as the proportion of vessels (n = 250) with each one. Chemical reactions of the host to fungus colonization or wounding were determined by evaluation of the presence of lipids, pectins and phenolics and increased lignification with stained sections. All observations were made with a light microscope at 200× or 400×.

3 Results

3.1 Symptom development

All control wounds were closed over with callus tissue, and no discoloration was observed in the sapwood (Fig. 2a). Cankers formed around each fungus inoculation point while no cankers occurred with water inoculation points 2 months after inoculation (Fig. 2b). The mean length of inner bark lesions associated with the cankers was significantly greater at 12 *vs.* 2 months (Table 1). Cankers expanded slowly in a lateral direction, but there was a significant increase in width of inner bark lesions between 2 and 12 months after inoculation (Table 1).

Long and narrow areas of reddish-brown discoloration of sapwood (= xylem lesions) were evident in the sapwood at all fungus inoculation points (Fig. 2c). In cross section, xylem lesions were wedge-shaped (Fig. 2d). At all fungus inoculation points, xylem lesions extended inward in a radial direction from the current year's annual ring through multiple older annual rings and reached the outer boundary of heartwood. Xylem lesions and inner bark lesions were similar in length (vertically) and both exceeded the length of the corresponding outer bark necrosis (Table 1). The vertical distance that xylem lesions extended above or below fungal inoculation points did not differ significantly. Two months after inoculation, the lesions extended 3.0–27.5 cm above and 4.0–23.5 cm below the inoculation points. After 12 months, xylem lesions extended 26.0–40.0 cm above and 26.0–39.0 cm below the inoculation points. Continuous xylem lesions were associated

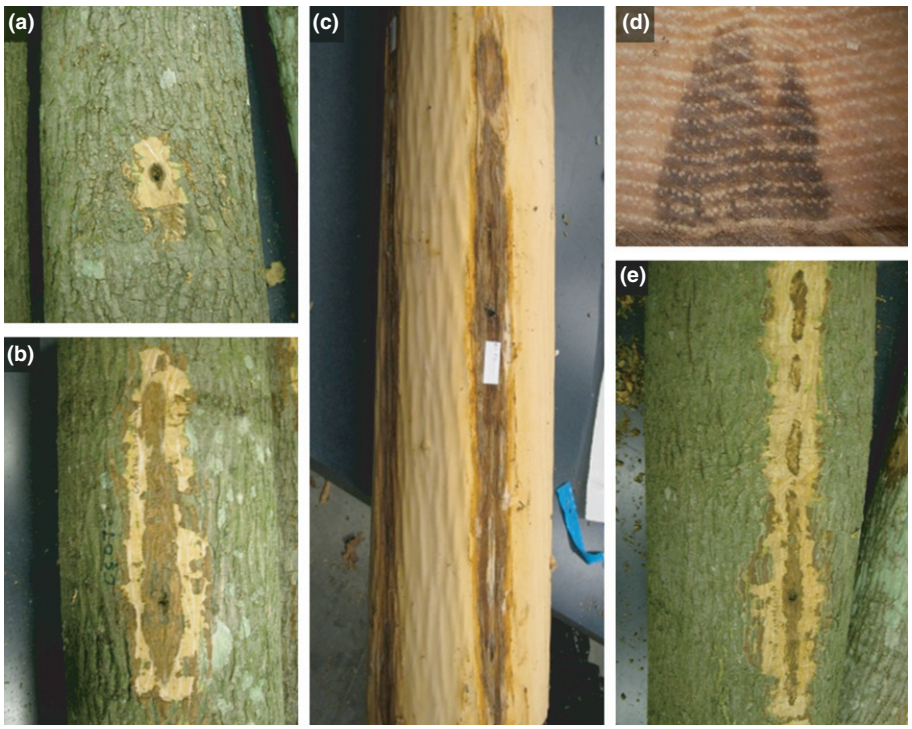

Fig. 2. Symptom development following *Ceratocystis smalleyi* inoculation of bitternut hickory stems. (a) Water-inoculated wound 2 months after inoculation. (b) Necrotic inner bark caused by *C. smalleyi* 2 months after inoculation. (c) Long, narrow discoloration of sapwood resulting from *C. smalleyi* inoculation 12 months later. (d) Wedge-shaped, reddish-brown sapwood discoloration associated with a fungus inoculation point 2 months after inoculation. (e) Four separate inner bark lesions formed on a stem section within 2 months following fungus inoculation.

Table 1. Average size of inner bark and xylem lesions caused by *Ceratocystis smalleyi* inoculation of bitternut hickory stems compared with water control.

Months after inoculation	Inoculum type	No. trees inoculated	Total no. inoculation points	Inner bark lesion width[1](cm)	Inner bark lesion length[1] (cm)	Xylem lesion length[1] (cm)
2	Water	2	8	0.2 ± 0.02 a	0.2 ± 0.02 a	NA[2]
2	*C. smalleyi*	4	16	2.6 ± 0.08 b	21.8 ± 2.64 b	23.6 ± 2.84 a
12	*C. smalleyi*	2	8	3.0 ± 0.10 c	64.2 ± 3.20 c	68.0 ± 3.27 b

[1]Values are means plus standard errors. Statistical differences indicated by different letters within each column were determined by Tukey's HSD test at the 95% confidence level.
[2]Not applicable as water inoculations did not induce xylem lesions in study trees.

with one to four distinct phloem lesions, characteristic of canker resurgence (Fig. 2e). Such resurgence was commonly observed with one (n = 13 of 24 inoculations) or two (n = 7) and seldom with three (n = 1) or four (n = 3) phloem lesions. *C. smalleyi* was recovered from all xylem tissues above and below the inoculation points, but not from xylem tissues associated with the control wounds.

3.2 Quantitative features

Fungal structures and vessel occlusions were observed in the outer 10 annual rings at the lateral margins of areas with sapwood discoloration at 2 and 12 months. Many fungal hyphae were observed in the discoloured sapwood area and advancing hyphae at the margin of the discoloured xylem. For all sections examined, no sporulation of *C. smalleyi* was observed. Hyphae were commonly observed in all types of xylem features such as axial parenchyma, ray parenchyma, fibres and vessels and were particularly abundant in the axial and ray parenchyma (Fig. 3a–c). Hyphae growing along large vessels were frequently branched in the vessel lumen (Fig. 3d). The primary passage of hyphae from cell to cell was through bordered pits, probably the result of penetration by mechanical pressure as seen by hyphae becoming thin in the pit regions (Fig. 3c and e). Hyphal projections were commonly observed protruding from ray parenchyma cells to vessel lumens (Fig. 3f).

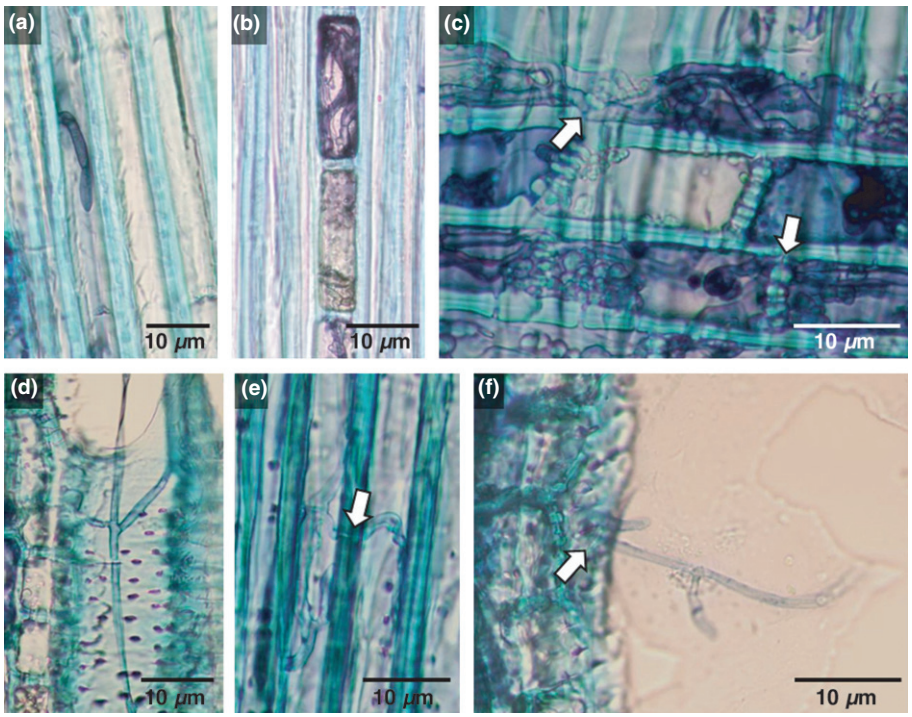

Fig. 3. The appearance of *Ceratocystis smalleyi* in colonized sapwood tissues of bitternut hickory following stem inoculation. Sections were stained with toluidine blue O. (a) Advancing hyphal tip in a wood fibre. (b) Hyphae in axial parenchyma cells. (c) Hyphal penetration (arrows) of bordered pits between radial parenchyma cells. (d) Branching of hyphae in a large vessel. (e) Hyphal penetration (arrow) of a pit aperture between wood fibres. (f) Hyphae (arrow) projecting from radial parenchyma cell to a vessel lumen. Fungal hyphae were observed in these xylem features at 2 and 12 months.

Tyloses formed abundantly in earlywood vessels of all fungus-inoculated trees (Fig. 4a) while gels partially or completely occluded latewood vessels (Fig. 4b and c). Most tyloses were thin-walled. Vessel lumens of earlywood vessels were normally closed by multiple, contiguous tyloses (Fig. 4d). When latewood vessels were occluded with gels, the same gel substances were observed abundantly in surrounding parenchyma cells (Fig. 4e).

Vessel occlusion occurred in response to both water inoculation and fungus inoculation, but it was more abundant in fungus-inoculated trees (Fig. 5). After 2 months, 36–70% (mean 54 ± 2.4%) of the vessels were occluded by tyloses or gels at the margin of the infected area. After 12 months, the percentage of occluded vessels (34–78%, mean 57.3 ± 2.5%) was slightly greater than that occurring after 2 months. Wound-induced tylosis formation occurred in 16–32% of vessels in water inoculation points with a single exception in the nearest point (point 1) of one inoculation site where 48% of vessels were plugged. Gel accumulation in a vessel did not occur in water-inoculated trees. The proportion of occluded vessels was consistent or increased by distance from the fungus inoculation points (Fig. 5b and c) while the proportion of wound-induced tyloses decreased by distance from the water inoculation points (Fig. 5a).

In the rest of the vessels that were non-occluded, fungal hyphae were observed at frequencies of 0.4–12.4% of vessels after 2 months and 0.4–34.7% of vessels after 12 months in fungus-inoculated trees. The mean proportion of fungus-colonized vessels was higher after 12 months (mean 8.1 ± 2.1%) than after 2 months (mean 2.1 ± 0.7%). Colonized vessels were more abundant near the point of inoculation and decreased as vertical distance increased from the inoculation wound (Fig. 5b and c). No fungal hyphae were observed in water-inoculated trees.

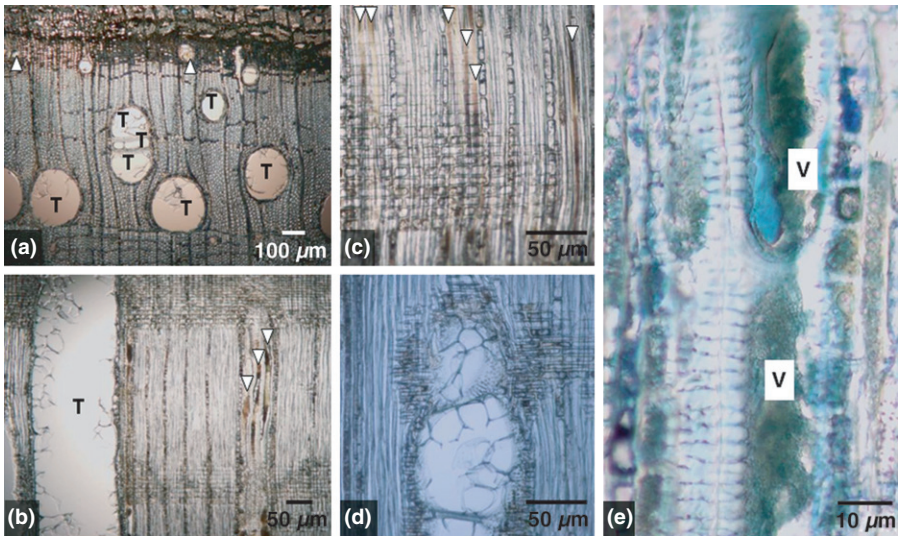

Fig. 4. Vessel occlusion by tyloses or gels in reaction zone in xylem of *C. smalleyi*-inoculated bitternut hickory. (a) Tyloses (T) in earlywood vessels and gels (arrow heads) in latewood vessels in transverse view. (b) Tyloses (T) in an earlywood vessel element and gels (arrow heads) in latewood vessels in radial view. (c) Latewood vessels occluded with gels in radial view (arrow heads). (d) Earlywood vessel elements showing contiguous tyloses in radial view. (E) Gels in latewood vessel elements and surrounding parenchyma cells in radial view. (a, d) Sections stained with Nile blue. (b, c) Sections without staining. (e) Section stained with toluidine blue O. These features were observed in the xylem at 2 and 12 months after inoculation.

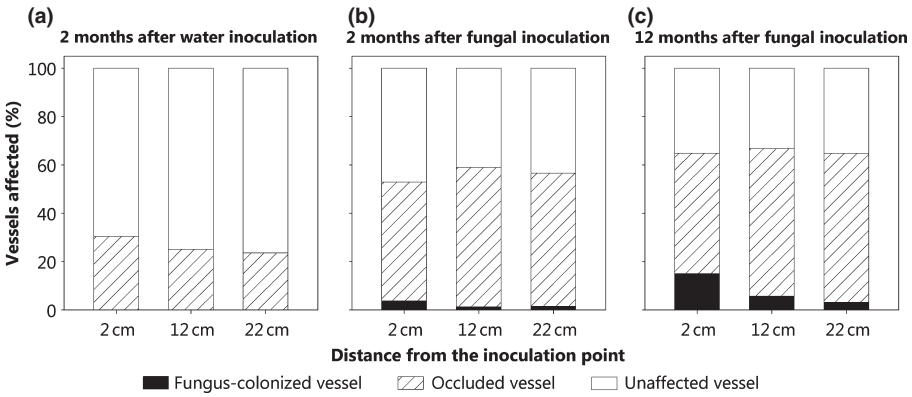

Fig. 5. Frequencies of unaffected, occluded by tyloses or gels, and fungus-colonized vessels at the margin of discoloured sapwood in bitternut hickory xylem 2 months (b) and 12 months (c) after inoculation with *Ceratocystis smalleyi* compared with those inoculated with sterile water (2 months only) (a). The results shown are means obtained from 8 replicates of fungus inoculation and 4 replicates of water control.

3.3 Qualitative features

Accumulated lipids, pectin deposits and phenolic compounds were observed consistently in sapwood samples taken at 2 and 12 months from the reaction zone in the outer 10 annual rings. In discoloured sapwood, parenchyma cells appeared necrotic based on the lack of any cell contents (Fig. 6a). In contrast, reserve starch was abundant in unaffected parenchyma cells in the sapwood of water-inoculated trees and in the unaffected sapwood of fungus-inoculated trees (Fig. 6b). At the margin of discoloured sapwood (reaction zone), lipid was detected (i.e. blue by Nile blue staining) and accumulated in the axial and ray parenchyma cells (Fig. 6c). More of the lipid content of parenchyma cells adjacent to vessels appeared to have leaked into the vessels through bordered pit apertures (Fig. 6d). This was more pronounced the nearer the parenchyma cell was to the discoloured area. In discoloured areas, cell contents were depleted in parenchyma cells surrounding vessels and the vessels were partially or completely occluded by gels (Fig. 6e).

Brownish darkening of cells commonly occurred in colonized tissues of phloem, cambium and xylem in the outer 10 growth rings of the sapwood taken from fungus-inoculated trees (Fig. 7a). In the xylem, this was more distinct in the ray parenchyma. In the controls, dark-brown discoloration was observed in some ray parenchyma, but it was limited to the area near the inoculation wound. Phenolic compounds were seldom detected by 4-nitrosophenol in fungus-inoculated tree tissues in the outer 10 annual rings. When observed, the phenolics were mostly present in axial parenchyma cells (Fig. 7b) and in a few latewood vessels that were occluded with gels in the advance of reaction zone (Fig. 7c). No phenolic compounds were observed in sapwood tissues of water-inoculated trees or in unaffected area of fungus-inoculated trees. Pectin materials were initially detected in axial parenchyma cells surrounding vessels in the reaction zone of fungus-inoculated trees (Fig. 7d). As sapwood tissues became more extensively colonized, pectin moved from the parenchyma cells and accumulated in the adjacent vessels (Fig. 7e). There was no accumulation of pectin in controls and unaffected sapwood tissues. No distinctive lignification of xylem cell walls was observed in the reaction zone.

4 Discussion

Extensive colonization of bitternut hickory by *C. smalleyi* was clearly visible in principal components of the axial structure of xylem at both 2 and 12 months after inoculation. Specifically, hyphae were observed in vessels, fibres and parenchyma cells in the reaction zone at the xylem lesion margin where host defence responses actively occur. The fungus was also abundant in radial parenchyma cells. This physical presence of *C. smalleyi*, particularly of advancing hyphae, in the reaction zone and high recovery rates of the fungus from xylem lesions at 2 and 12 months after inoculation indicate that *C. smalleyi* has the ability to invade, colonize and remain viable in xylem tissues for at least 1 year. Hyphal projections from radial parenchyma cells into vessel lumens and hyphae growing along vessels were commonly observed. These features may be related to the radial and vertical spread of the fungus in xylem cells that could likely lead to the expansion of xylem lesions

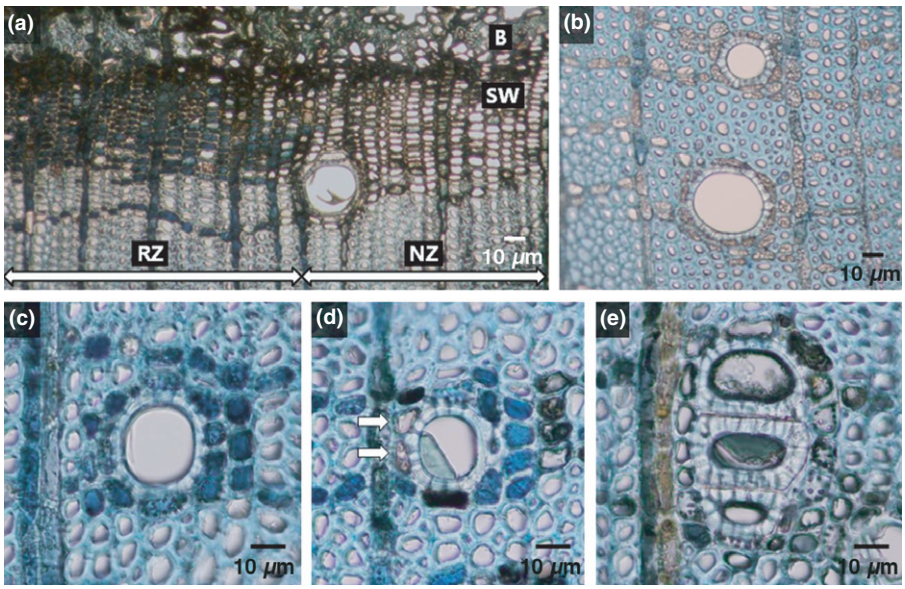

Fig. 6. Lipid accumulation in reaction zone in xylem of *C. smalleyi*-inoculated bitternut hickory. (a) Radially aligned lipid-rich parenchyma cells in reaction zone (RZ) at the margin of discoloured sapwood. Parenchyma cells in necrotic zone (NZ) depleted their cell contents. B: bark, SW: sapwood. (b) Unaffected parenchyma cells in non-discoloured tissue containing abundant starch. (c) Lipid accumulation in parenchyma cells in reaction zone of a fungus-inoculated tree. (d) Lipid depletion in parenchyma cells (arrows) and an adjacent vessel partially occluded with gel in discoloured sapwood. (e) Necrotic parenchyma cells with depleted cell contents and vessels occluded with gels in discoloured sapwood. (a-e) Transverse sections stained with Nile blue. These features were observed in the xylem at 2 and 12 months after inoculation.

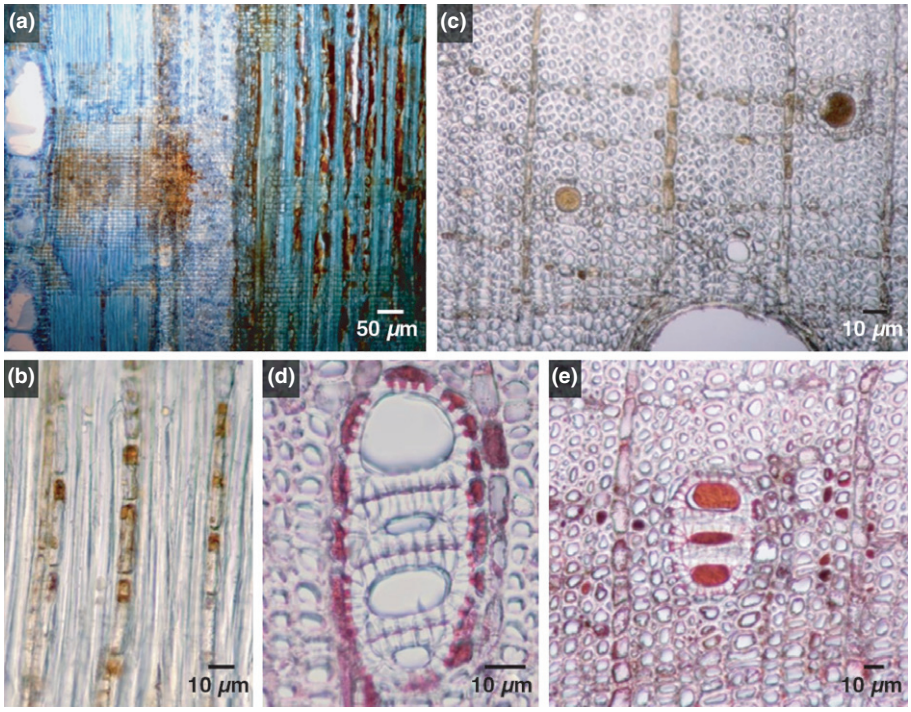

Fig. 7. Browning of cells and accumulation of phenolic and pectic compounds in reaction zone in xylem of *C. smalleyi*-inoculated bitternut hickory. (a) Brown discoloration of cells in colonized phloem, cambium and ray parenchyma of xylem in radial view. (b) Phenolic compounds concentrated near end walls of axial parenchyma cells in radial view. (c) Phenolic compounds detected in latewood vessels. (d) Pectin materials in surrounding parenchyma cells of latewood vessels. (e) Pectin materials mobilized and accumulated in latewood vessels and wood fibres in discoloured wood. (a) Section stained with toluidine blue O. (b, c) Sections stained with 4-nitrosophenol. (d, e) Sections stained with ruthenium red. These features were observed in xylem 2 and 12 months after inoculation.

in both directions. Hyphal growth from cell to cell occurred exclusively through bordered pits similar to that reported for *Fusarium oxysporum* f.sp. *perniciosum* invading mimosa trees (Phipps and Stipes 1976). Hyphal constriction seemed to be the means by which the fungus grew from cell to cell based on observation of thin hyphae in the border pits of such cells. Fungal penetration by mechanical pressure was observed for *Chalara fraxinea* infecting ash trees (Schumacher et al. 2010). However, neither intercellular growth in middle lamella nor direct penetration through cell walls was found.

No conidiophores or conidia were observed in xylem features in the reaction zone of *C. smalleyi*-infected bitternut hickory at 2 or 12 months. In contrast, true wilt pathogens produce both conidia and hyphae when colonizing host sapwood, for example, *Ceratocystis fagacearum* in oak (Sinclair and Lyon 2005) and *F. oxysporum* f.sp. *perniciosum* in mimosa (Phipps and Stipes 1976). *In situ* production of spores is crucial for establishment of such wilt pathogens in the xylem because spores can be transported rapidly throughout the vascular system while invasion by hyphae occurs slowly. Observation of only fungal hyphae in inoculated hickory is similar to the Japanese oak wilt situation where only hyphae of *Raffaelea quercivora* have been observed in infected oaks (Takahashi et al. 2010). The dependency on hyphal growth for advancing in the xylem may be one of the factors accounting for why both *C. smalleyi* and *R. quercivora* do not spread systemically throughout their hosts. The general defence responses detected in *C. smalleyi*-infected xylem are consistent with microorganism colonization of other hosts. Vessel occlusion by tyloses or gel deposits is a general defence mechanism of angiosperms associated with sapwood infection. This response is considered to limit pathogen progression within vessels as well as water loss from the area around damaged tissue (Pearce 1996; Rioux et al. 1998; Sun et al. 2008). In general, plants respond to infection by producing either tyloses (e.g. *Ulmus hollandica*) or gels (e.g. *Prunus pensylvania*) (Pearce 1990; Yamada 2001; Sun et al. 2008). In contrast, both tyloses and gels were produced in response to *C. smalleyi* invasion. The observation of abundant pectic substances in parenchyma cells, particularly near vessels, of the reaction zone in *C. smalleyi*-infected bitternut hickory is not an unusual phenomenon. Production of vascular gels is common in other wounded or infected trees (Rioux et al. 1998; Sun et al. 2008). Pectic substances appear to be secreted from parenchyma cells into adjoining vessels through pits, thus partially or completely clogging the vessel. Phenolic compounds were detected in a few of the occluded vessels of infected bitternut hickory, similar to that observed in other trees species (e.g. cricket bat willow, royal palm trees) (Wong and Preece 1978; Weiner and Liese 1995).

Lipid-rich parenchyma cells in the reaction zone were distinctive from either healthy, unaffected cells, which contained abundant starch grains or necrotic colonized cells where all cell contents were depleted. Such increase in lipid in parenchyma cells has been observed in association with other tree diseases (e.g. pine wilt, Japanese oak wilt) (Yamada et al. 2003; Hara et al. 2006). Hara et al. (2006) found that lipid in parenchyma cells diffuses into surrounding tracheids as pinewood nematode invasion proceeds in susceptible pine trees based on observations of samples obtained successively over

time and by distance from the inoculation point. This transitioning of parenchyma cell composition agrees with our findings for the bitternut hickory–*C. smalleyi* interaction. Lipid response occurs as a consequence of cell damage or of senescence or from an active defence response in host–microbial interaction (Newcombe and Robb 1989). In the latter case, lipid contributes to formation of an impermeable barrier by being incorporated into coating materials (Bishop and Cooper 1983; Newcombe and Robb 1989). It is likely that the lipid response induced in bitternut hickory plays an important role in limiting further penetration of *C. smalleyi* in xylem unless the fungus produces abundant lipases like some other fungi and bacteria (Sztajer et al. 1988; Singh and Mukhopadhyay 2012).

Phenols were rarely detected in a few axial parenchyma cells in the advance of the reaction zone. Phenolic compounds are known to act as constitutive components of sapwood or as phytoalexins (Pearce 1996). In either case, however, phenols create an inhibitory environment for fungal growth. Further investigation is needed to determine whether the phenols detected in bitternut hickory are functioning as phytoalexins. Even though this may be the case, their fungistatic effects were not clear because the distribution of detected phenols was sparse.

The lesion development in the phloem and xylem seemed to reflect the manner in which *C. smalleyi* invades the outer sapwood and the accompanying host defence responses. Xylem lesions more readily extended in a vertical and radial direction than in a lateral direction based on this study and personal observations. Although it is possible that fungal spores inoculated into the outer xylem were forcibly drawn up via the transpiration stream in vessels, no spores were found in the xylem tissues examined. It is also possible that *C. smalleyi* hyphae invade through vertical and radial routes more rapidly than lateral ones even though their spread may occur in all directions. In terms of wood structure, both pathways (axially along vessels and radially in the rays) are easier routes for fungal spread compared with the lateral movement from vessel to vessel (Pearce 1989). Nevertheless, *C. fagacearum*, a true vascular pathogen, can successfully spread laterally and longitudinally into many vessels in susceptible red oak (Jacobi and MacDonald 1980). The limited lateral progression of *C. smalleyi* in bitternut hickory xylem is consistent with the localized lesions, that is, wedges of discoloured sapwood, observed in cross sections of infected stems.

Xylem lesions extended further vertically than their corresponding lesions in the phloem. A long, continuous xylem lesion corresponding to several bark lesions was commonly observed. This phenomenon has been observed commonly in other hardwood diseases (Panconesi 1999; Brown and Brasier 2007; Schumacher et al. 2010). Its occurrence within hickory may indicate *C. smalleyi* has the ability to progress outward from the xylem to the cambium and phloem, thus initiating a new bark canker. This habit of *C. smalleyi*, together with abundant hyphae and its viability for at least 1 year in the xylem, supports the hypothesis that xylem tissues are the preferred substrate of the fungus over phloem tissues. However, this question requires further investigation.

The extent of pathogen colonization in xylem depends on the effectiveness of host resistance mechanisms to limit pathogen spread. Of the various host defence responses reported, Beckman (1966) emphasized the important role of vascular occlusion with gels or tyloses in sealing off the infected area and screening out migrating fungal structures. Such occluded vessels were extensively found in infected bitternut hickory. However, the occurrence of fungal hyphae colonizing up to 35% of otherwise unaffected vessels in the reaction zone may indicate that vessel occlusion was not able to completely restrict the progressing fungal colonization. Subsequent spread of the fungus may have resulted in expansion of xylem lesions over time. For pine wilt disease, Hara et al. (2006) found that the failure of initial defence responses to contain the pathogen and the excessive responses that followed further pathogen invasion contributed to the mortality of pines infested with pinewood nematode. Jacobi and MacDonald (1980) found that white oaks tolerant to *C. fagacearum* exhibited severe wilt symptoms when 50–60% of vessels were occluded following infection. Hydraulic dysfunction observed in *C. smalleyi*-infected trees (Park et al. 2013) could be the consequence of bitternut hickory's vigorous response to xylem infection by *C. smalleyi*, that is, tylose formation and gel accumulation in the vessels. Both responses appear to contribute to hydraulic dysfunction, thus, promoting foliar wilt in the tree crown.

The larger question of how and why *C. smalleyi* is restricted in the host compared with true vascular wilt pathogens remains unanswered. Jacobi and MacDonald (1980) suggested three factors that likely contribute to successful host colonization by *C. fagacearum*: (i) the production of small conidia that can be transported rapidly throughout the functioning xylem, (ii) the common occurrence of natural vessel anastomosing and (iii) the apparent lack of restriction to lateral growth of *C. fagacearum*. Among these factors, the first and third differ from observations in the *C. smalleyi*–bitternut hickory interactions. Unfortunately, the available information on vessel structure and wood anatomy of bitternut hickory is insufficient to evaluate the second factor. No sporulation was observed in the reaction zone of *C. smalleyi*-infected bitternut hickory and the principal means of *C. smalleyi* xylem invasion was via hyphal growth. Of the three dimensions observed, lesion development was restricted laterally in bitternut hickory suggesting lateral colonization of *C. smalleyi* is limited. It is probable that lipid-rich parenchyma cells that are radially aligned along the reaction zone form an effective barrier limiting lateral invasion of the pathogen. Suberized walls of parenchyma cells have lipid incorporated into them and constitute a structural barrier to the lateral and radial invasion of other pathogens in reaction zones (Newcombe and Robb 1989; Pearce 1996; Yamada 2001; Yamada et al. 2003). Another factor that should be considered is the involvement of cell degrading enzymes such as extracellular pectolytic or cellulolytic enzymes. These degrading enzymes are particularly beneficial for directly penetrating host cells as well as for dissolving the materials making up a defence barrier (Dimond 1970). If *C. smalleyi* lacks or is deficient in such extracellular enzymes, this may account for the pathogen's limited ability to overcome host defence responses and spread throughout the xylem like true wilt pathogens. Additional investigation of this point is warranted.

In conclusion, this study showed *C. smalleyi* has the ability to invade and colonize all types of sapwood xylem tissue of bitternut hickory following artificial inoculation. This indicates *C. smalleyi* is not only a phloem pathogen causing bark

lesions (Park et al. 2010), but also a sapwood pathogen causing xylem lesions that impair physiological functioning of the vascular system (Park et al. 2013). Bitternut hickory activates general defence responses following xylem infection by *C. smalleyi*. These responses include vessel occlusion with gels or tyloses, lipid accumulation and production of phenolic compounds. Pectic substances, lipids and, to a rare extent, phenolics detected in vascular gels are likely derived from vasicentric parenchyma cells. *C. smalleyi* colonization progresses gradually, occurring primarily in a vertical direction in spite of the activated host defence responses. The lack of fungus sporulation within vessels and the unsuccessful lateral colonization of *C. smalleyi* are factors that likely limit disease development to local xylem tissues compared with systemic spread of true vascular wilt pathogens in susceptible hosts.

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