Histological and anatomical responses in avocado, *Persea americana*, induced by the vascular wilt pathogen, *Raffaelea lauricola*

Sharon Inch, Randy Ploetz, Benjamin Held, and Robert Blanchette

Abstract: *Raffaelea lauricola* causes laurel wilt of avocado, *Persea americana*. Host × pathogen interactions were examined with light and scanning electron microscopy. The susceptible avocado cultivar 'Simmonds' was inoculated and examined 5 cm above the inoculation site 3, 7, 14, 21, and 42 days after inoculation (dai). No external symptoms were observed at 3 and 7 dai, and there were no anatomical differences when compared with the mock-inoculated plants. By 14 dai, external symptoms were present and dark discoloration had developed in sapwood. Tylose development increased significantly by 14 dai, and was positivity correlated with disease severity ($P < 0.05$). By 14 dai, gels formed in xylem vessels, fibers, and adjacent parenchyma cells; they were associated with xylem blockage and composed of phenols, pectin, and lipids, as suggested by, respectively, toluidine blue O, ruthenium red, and Sudan III stains. With a chitin-specific stain, fluorescein-conjugated wheat germ agglutinin, infrequent mycelia, and conidia of *R. lauricola* were visualized within xylem lumena and fibers, regardless of sample date. Understanding how avocado responds to the presence of this pathogen could assist the development of laurel wilt-resistant avocado genotypes and inform efforts to manage this disease with other measures.

Key words: avocado, laurel wilt, *Raffaelea lauricola*, *Xyleborus glabratus*, ambrosia beetle.

Résumé : Le *Raffaelea lauricola* cause la flétrissure du laurier chez l'avocat. Les auteurs ont examiné les interactions hôte × pathogène à l'aide de la microscopie photonique et électronique par balayage. Ils ont inoculé le cultivar d'avocat 'Simmonds' susceptible, et ont examiné la partie située à 5 cm au dessus du site d'inoculation, 3, 7, 14, 21, et 42 jours après l'inoculation (jai) On n'observe aucun symptôme aux jai 3 et 7, et il n'y a pas de différences anatomiques lorsque que l'on compare avec les plants inoculés à blanc. Vers le jai 14, on observe des symptômes externes montrant une décoloration foncée se développant dans l'aubier. Le développement des thylles augmente significativement vers le jai 14 et montre une corrélation avec la sévérité de la maladie ($P < 0{,}05$). Vers le jai 14, des gels se forment dans les vaisseaux du xylème, les fibres et les cellules de parenchymes adjacentes; ils sont associés au blocage du xylème et sont composés de phénols, pectine et lipides, tel que suggéré par les colorations au bleu de toluidine O, au rouge de ruthénium, et au Soudan III. Indépendamment de la date d'échantillonnage, on observe à l'aide d'un colorant spécifique à la chitine, une fluorescéine conjuguée à l'agglutinine du germe de blé, une présence peu fréquente de mycéliums et de conidies du *R. lauricola* dans les lumens du xylème et des fibres. Une meilleure compréhension des réactions de l'avocat à la présence de ce champignon pathogène pourrait aider à développer des génotypes d'avocat résistants à cette flétrissure et orienter les efforts d'aménagement de la maladie vers d'autres directions.

Mots-clés : avocat, flétrissure du laurier, *Raffaelea lauricola*, *Xyleborus glabratus*, scolyte du bois.

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Introduction

Laurel wilt affects American members of the Lauraceae plant family, including the important fruit crop avocado (*Persea americana* Mill.) and native trees in the southeastern USA, such as redbay (*Persea borbonia* (L.) Spreng.) and swampbay (*Persea palustris* (Raf.) Sarg.) (Fraedrich et al. 2008; Ploetz et al. 2011*a*). The disease is caused by *Raffaelea lauricola* (T.C Harr., Fraedrich & Aghayeva), an asexual fungal symbiont of a non-native Asian ambrosia beetle, *Xyleborus glabratus* (Eichhoff), that was most likely transported in maritime packing materials to Port Wentworth, Georgia, USA, the first location it was detected in the Western Hemisphere (Fraedrich et al. 2008).

Fungal symbionts of ambrosia beetles are carried in specialized structures, mycangia (Rabaglia et al. 2006). Female ambrosia beetles bore into host trees to create brood galleries in which they cultivate gardens of the symbionts, which in turn feed adults and larvae of the insect (Harrington 2005).

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Since *X. glabratus* was first detected in May 2002, laurel wilt has spread throughout the southeastern USA; it is now found in Florida, Georgia, Mississippi, North Carolina, and South Carolina (USDA 2011). In 2011, laurel wilt was reported on swamp bay in Miami-Dade County, 6 km from the main avocado-producing areas in Florida (Ploetz et al. 2011*b*). Potential losses that laurel wilt could cause to avocado production in Florida have been estimated at US$27–54 million (Evans et al. 2010).

Symptoms of laurel wilt, on avocado, redbay, and swampbay, include rapid wilting of foliage and vascular discoloration. Blockage of the water conducting system appears to be at least partially responsible for the wilting symptoms (Inch and Ploetz 2011). Wilting and vascular discoloration are typically associated with vascular wilt diseases. These symptoms have been attributed to physical and histological changes (Beckman 1964), which include the production of gels (Bonsen and Kucera 1990) and tyloses (Bishop and Cooper 1984) in the host. Elm (Rioux and Ouellette 1989; Rioux et al. 1998), oak (Struckmeyer et al. 1954; Jacobi and MacDonald 1980), and grape (Fry and Milholland 1990) are woody hosts for which such responses have been reported. Gels and tyloses may confine and prevent the systemic spread of pathogens (Yamada 2001).

Tyloses are formed in xylem lumena in response to infection, embolism, aging, and injury (Rioux et al. 1998). They are out-growths from adjacent parenchyma cells, and prevent desiccation, damage or infection of adjacent cells (Rioux et al. 1998). Plugging of xylem vessels by tyloses has been found to reduce hydraulic conductivity in vivo (Collins et al. 2009).

Gels induced by pathogens generally arise from host perforation plates, end walls, and pit membranes of the primary wall and middle lamella (VanderMolen et al. 1977; Aist 1983; Rioux et al. 1998). Breakdown of these cellular components by the pathogen results in the accumulation of gels (Gagnon 1967). The chemical nature of gels has been examined with histochemical stains (Jensen 1962); in general, they are composed of lignin, pectin, and phenolics (Ouellette 1980; Gagnon 1967).

Degradation of vessel walls has been observed in *Ulmus americana* infected by *Ophiostoma ulmi*. *Ophiostoma ulmi* produces extracellular cell-wall degrading enzymes (Beckman 1964; Dimond and Husain 1958). In *U. americana* artificially inoculated with this pathogen, compounds were deposited in vessels that stained positive for lignin and pectins (Gagnon 1967). Pectic substances have also been observed in xylem vessels of tomato infected with *Fusarium oxysporum* f. sp. *lycopersici* (Ludwig 1952), most likely owing to the production of pectinases by the pathogen (Waggoner and Dimond 1954). Although gels and tyloses have been observed in avocado infected with *R. lauricola* (S. Inch et al. unpublished), nothing is known about their composition.

Disruption of water conduction is very common in plants affected by vascular wilt pathogens (Beckman et al. 1953). Xylem dysfunction and impaired water transport has been observed in *R. lauricola*-infected avocados, but the cause(s) of these changes is not known (Inch and Ploetz 2011). Although plugging of xylem with bacterial cells has been indicted in bacterial vascular wilts (Husain and Kelman 1958; Fry and Milholland 1990; Chatterjee et al. 2008), colonization of xylem vessels by fungal vascular wilt pathogens is generally insufficient to cause wilting (Beckman 1964; Waggoner and Dimond 1954).

Chitin-specific, fluorescein-conjugated wheat germ agglutinin (F-WGA) stains the fungal cell walls of many ascomycetes, including *Raffaelea quercivora* (Takahashi et al. 2010) and *F. oxysporum* (Schoffelmeer et al. 1999). With this stain and epifluorescence microscopy, Takahashi et al. (2010) visualized the distribution of *R. quercivora* in artificially inoculated oak trees (*Quercus serrata* and *Quercus crispula*). Little is known about the temporal and spatial distribution of *R. lauricola* in infected avocado (Inch and Ploetz 2011).

Recently, external (wilting and foliar necrosis) and internal (sapwood discoloration) symptom development was examined in grafted avocado plants that were artificially inoculated with *R. lauricola*. Significant differences were observed among different cultivars of this crop. Cultivars with a West Indian background, such as 'Simmonds', were highly susceptible. Where as cultivars with a Guatemalan or Mexican background tended to be less susceptible (Ploetz et al. 2011*c*). There are no resistant varieties available at this time. Disease severity was positively correlated with plant size, and significant symptoms developed internally before external symptoms were evident.

In the present study, the response of avocado ('Simmonds') to artificial infection by *R. lauricola* was examined with light and scanning electron microscopy. With the F-WGA stain, the internal distribution of the pathogen was examined in the host over time. In addition, we determined anatomical and histochemical changes that were induced by *R. lauricola* in this host, and the temporal correlation of these changes with the development of laurel wilt symptoms.

Materials and methods

Potted trees of the susceptible avocado cultivar 'Simmonds' were obtained from a commercial nursery and maintained in an air-conditioned greenhouse (ca. 25 °C) at the University of Florida's Tropical Research & Education Center (TREC) in Homestead, Florida, USA. Plants were clonal scions of 'Simmonds' grafted on seedling rootstocks, and were in 12-L pots, 1–1.5 m in height, with main stems about 3 cm in diameter. Standard irrigation and fertilization practices were utilized (Ploetz et al. 2011*c*).

An isolate of *R. lauricola* (RL4) that had been used in previous disease trials and deposited at the Centraalbureau voor Schimmelcultures (CBS Fungal Biodiversity Centre, Utrecht, the Netherlands) (CBS 127349) was used in this study (Ploetz et al. 2011*c*). Conidia were harvested from 7-day-old malt extract agar cultures by flooding with sterile, distilled water and gently scraping the surface with sterile glass rods. Prior to use the final spore concentration was adjusted to 1×10^6 conidia mL^{-1}.

Approximately 15 cm above the graft union, a 2-mm-diameter hole was drilled at a 45° downward angle, and 100 µL of the conidial suspension or sterile water (mock inoculation) was pipetted into the hole, which was then sealed with Parafilm. At 3, 7, 14, 21, and 42 days after inoculation (dai) and 42 days after control plants were mock-inoculated, plants were rated externally and internally for laurel wilt severity and processed for light and scanning electron micro-

scopy. For each of the six treatments (3, 7, 14, 21, and 42 dai and mock-inoculated), there were four single plant replicates. The experiment was conducted twice, and a completely randomized experimental design was used in both experiments.

Disease ratings

External and internal disease severity was estimated based on a subjective rating scale of 1 to 10, where 1 = no symptoms; 2 = 1%–11% of the canopy or sapwood symptomatic; 3 = 12%–23%; … 9 = 88%–99%; and 10 = dead or completely symptomatic (Ploetz et al. 2011*c*). Internal disease severity, based on percentage of sapwood discoloration, was assessed after bark from the main stem had been removed.

Recovery of *R. lauricola*

For each treatment replicate, six pieces of tissue (5 mm^3) were taken from 5 cm above the inoculation point and at 20 cm intervals thereafter. Tissue pieces were surface disinfested for 15 s in 70% ethanol, followed by 2 min in 10% bleach, rinsed in sterile water, blotted dry, and plated on a semi-selective medium, CSMA+ (Ploetz et al. 2011*b*). The plates were incubated on a laboratory bench under low light at 25 °C. After 10 days, the number of pieces from which *R. lauricola* was recovered was recorded.

Light microscopy

From each treatment replicate, 10 sections, 1 cm^3 in size and 5 cm above the inoculation point, were fixed in formalin – acetic acid – 50% ethanol (FAA) (5:5:90). After at least 48 h of fixation, samples were dehydrated in a series of ethanol and tertiary butyl alcohol solutions for 1 h each prior to embedding in paraffin (Paraplasts Plus). Thin sections (10 μm) were cut from embedded samples with a rotary microtome (American Optics model 820). To remove paraffin prior to staining, sections were washed in xylene and dehydrated in a series of ethanol solutions.

Three stains (Jensen 1962) were used to examine the chemical nature of gels that were deposited in xylem vessels. To detect pectic compounds, sections were stained with aqueous ruthenium red (1:5000) for 20 min. For lipid detection, fresh sections were placed in 50% ethanol for 3 min, transferred to a saturated and filtered solution of Sudan III in 70% ethanol for 20 min, and then differentiated in 50% ethanol for 1 min. To detect phenolic compounds such as lignin, tannins, and pectins, a drop of 0.05% aqueous toluidine blue O was placed on sections for 2–4 min and then rinsed in distilled water.

To visualize *R. lauricola*, samples were taken from each treatment replicate during both experiments. At about 5 cm above the inoculation point, 90 μm-thick sections were cut with a sliding microtome and fixed for 7 days (20% formalin, 10% dimethylsulfoxide, 0.1% Nonidet P-40, and 0.1 $mol \cdot L^{-1}$ sodium phosphate buffer (SPB) (pH 7.0)). Samples were washed three to four times with 0.1 $mol \cdot L^{-1}$ SPB to remove fixative, immersed in 10% KOH for 20 min at room temperature, and washed three times with 0.1 $mol \cdot L^{-1}$ SPB. Sections were then incubated in 2% bovine serum albumin (BSA) in 0.1 $mol \cdot L^{-1}$ SPB for 1 h, washed three times with 0.05% Tween 20 in 0.1 $mol \cdot L^{-1}$ SPB, immersed in 0.01% F-WGA (Sigma) solution (0.01% F-WGA, 1% BSA, and 0.1 $mol \cdot L^{-1}$ SPB) for 3 h, and washed three times with 0.05% Tween 20 in 0.1 $mol \cdot L^{-1}$ SPB. Stained sections were mounted on glass slides with Mowiol mounting medium (25% glycerol, 10% Mowiol 4–88, 5% 1,4-diazabicyclo(2.2.2)octane, 0.1 $mol \cdot L^{-1}$ tris-HCl (pH 9.0)) (Takahashi et al. 2010), viewed with an epifluorescence microscope (Nikon Optiphot) with an excitation of 460–490 nm, emission of 510 nm and photographed with a Canon EOS Rebel SLR digital camera.

Tylose formation was observed for treatment replicate about 5 cm above the inoculation point. Thick sections (90 μm) from frozen stems were cut with a rotary microtome (American Optics model 820), placed on glass slides, stained for 1 min with toluidine blue O, rinsed, and viewed with a light microscope (Nikon Optiphot). In a predefined 5 mm × 5 mm area, the numbers of xylem vessels and xylem vessels with tyloses were counted. The percentages of xylem vessels with tyloses were then calculated as:

$$\frac{\text{Number of xylem vessels with tyloses}}{\text{Total number of xylem vessels}} * 100$$

Data from the two experiments were pooled prior to mean separation with the Tukey–Kramer HSD statistic (SAS Institute Inc. 2011) (Table 1). A polynomial regression analysis was conducted to examine the relationship between percentage of vessels with tyloses and dai. To elucidate general trends in these data, a correlation matrix was constructed with dai, external severity, internal severity, and percentages of xylem vessels with tyloses (Table 2). Data were log transformed prior to statistical analysis.

Table 1. Mean responses of 'Simmonds' avocado stems and percentages of xylem vessels in which tyloses formed.

Days after inoculation	External severity	Internal severity	Percentage of tyloses
3	1.0a	1.0a	1.5a
7	1.0a	2.4ab	1.7a
14	2.7a	3.6b	22.9b
21	6.3b	7.4c	39.6c
42	8.0b	8.5c	51.4c
Mock inoculated	1.0a	1.0a	1.4a

Note: Potted 'Simmonds' avocado trees were inoculated with *Raffaelea lauricola* and examined microscopically for tylose formation 5 cm above the inoculation point after the indicated time intervals. Data are means for prescribed 25 mm^2 areas in each of four replicate trees and two experiments (eight total stem sections). Mock-inoculated (water) plants were sampled 42 days after inoculation. Disease was assessed externally (external severity) and internally (internal severity) on a 1–10 subjective scale. Within columns, means followed by different letters are significantly different according to Tukey–Kramer HSD ($P \leq 0.05$).

Table 2. Correlation matrix of days after inoculation (DAI), external symptoms (ES), internal symptoms (IS), and percentage of xylem vessels with tyloses (T%).

	ES	IS	DAI	T%
ES	1			
IS	0.9274	1		
DAI	0.8736	0.9026	1	
T%	0.9706	0.9183	0.8727	1

Note: All correlation coefficients are significant, $P \leq 0.0001$.

Fig. 1. Light micrographs of 'Simmonds' avocado: (A) transverse section from mock-inoculated control, in which no tyloses are evident in xylem vessels; and (B) transverse section stained with toluidine blue O, 42 days after inoculation (dai) with *Raffaelea lauricola*, in which numerous tyloses are evident. Scanning electron micrographs of 'Simmonds' avocado: (C) transverse; and (D) longitudinal sections 42 dai show nearly complete occlusion of vessels. Arrows in (B), (C), and (D) locate tyloses (T). Scale bars = 50 μm.

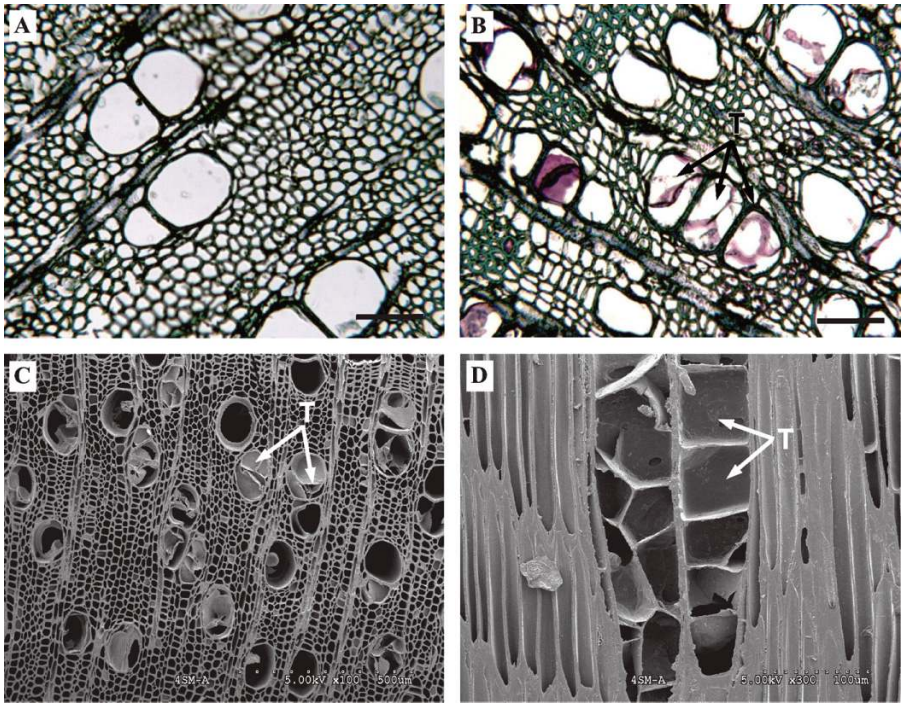
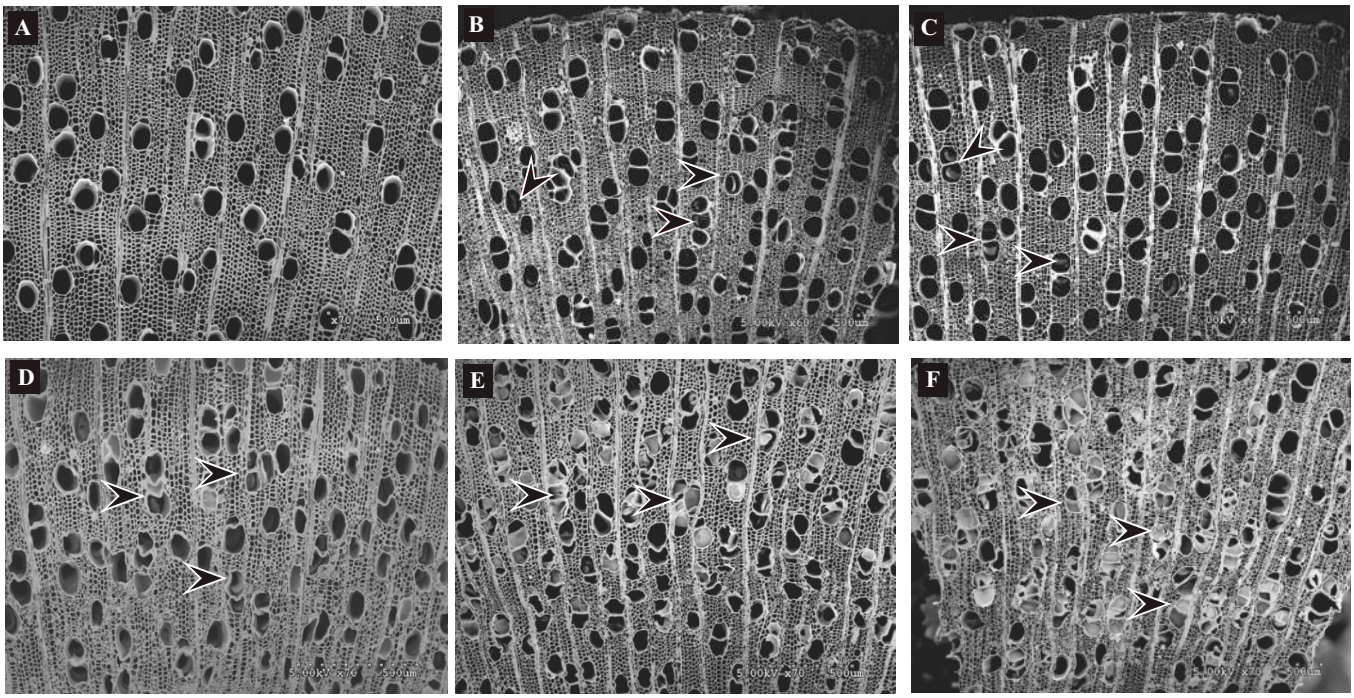


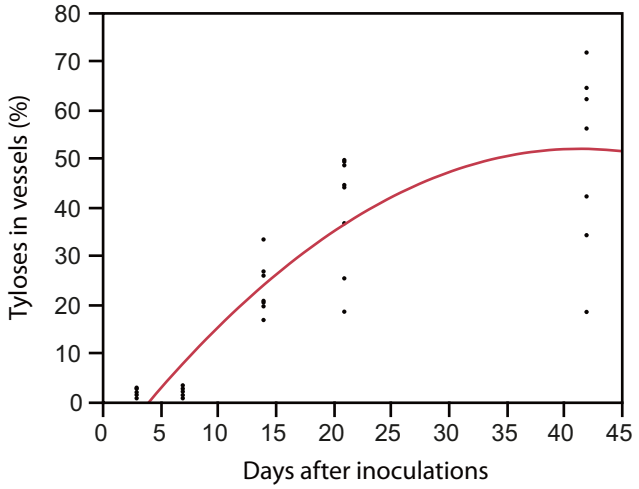
Fig. 2. Scanning electron micrographs of transverse sections from 5 cm above the inoculation point of (A) mock-inoculated and *Raffaelea lauricola*-inoculated 'Simmonds' avocado stems at (B) 3 days after inoculation (dai), (C) 7 dai, (D) 14 dai, (E) 21 dai, and (F) 42 dai. Note the increase in tylose (arrows) formation over time in the inoculated stems.



Scanning electron microscopy

Duplicate samples were recovered from the same plants and location that were assessed for light microscopy. Sections, 1 cm^3 in size, were placed in 2% (*v*/*v*) glutaraldehyde in 0.1 $mol \cdot L^{-1}$ phosphate buffer (pH 7.2) for 20 h at 4 °C. Samples were then placed in phosphate buffer and shipped from TREC to the University of Minnesota (UM). At UM, samples were infiltrated in 25% TBS tissue freezing medium (Triangle

Fig. 3. Polynomial regression of the relationship between percentages of tyloses in xylem vessels (25 mm^2) formed in stems, 5 cm above the inoculation point, of 'Simmonds' artificially with *Raffaelea lauricola* and days after inoculation. $R^2 = 0.78$, $y = -0.69 + 1.79x - 0.04(x-17.4)^2$, $P < 0.0001$.



Biomedical Sciences, Durham, N.C., USA) under partial pressure, and mounted on brass stubs at –20 °C in an OM 2488 Minotome microtome-crystat (International Equipment Company, Needham Heights, Mass., USA). Frozen sections were then cut transversely and radially to create clean viewing faces, thawed, rinsed with water, and air-dried for 48 h. The specimens were mounted with carbon tape to aluminum stubs and coated with gold/palladium in an EMS 76M Ernest Fullum sputter coater (Ernest F. Fullam, Inc., Schenectady, N.Y., USA). Samples were examined with a Hitachi S3500N (Hitachi, Tokyo, Japan) scanning electron microscope.

Results

By 3 dai, *R. lauricola* was recovered on CSMA + 5 cm above the inoculation point, and at 7, 14, 21, and 42 dai was recovered from the entire length of the stem (data not shown). The pathogen was not isolated from mock-inoculated plants.

Numerous tyloses formed in the vessel elements of inoculated plants (Figs. 1B–1D), whereas vessels in mock-inoculated plants were relatively tylose-free (Fig. 1A). Irregular xylem vessel walls were observed in inoculated stems 42 dai (Fig. 1B) compared with those from mock-inoculated plants, which had round, well-defined vessel walls (Fig. 1A). No anatomical differences were observed in inoculated versus mock-inoculated plants at 3 and 7 dai (Figs. 2A and 2B). However, as dai and disease severity increased, anatomical differences were observed in transverse stem sections of inoculated plants (Figs. 2C–2F). By 21 and 42 dai, xylem vessels and fibers in secondary wood were degraded (Figs. 2E and 2F).

Percentages of vessels with tyloses increased exponentially as dai increased. During the first 7 dai there was a delay in the development of tyloses followed by a rapid increase. After 21 dai, the rate of tyloses formation decreased (Fig. 3). Minor tylose formation (<2%) was observed 3 and 7 dai, and in control plants 42 days after mock inoculation (Table 1). However, by 14 dai there was a significant increase in tylose development, compared with 3 and 7 dai, and by 21 and 42

Fig. 4. Scanning electron micrographs of longitudinal sections from (A) mock-inoculated and (B) and (C) *Raffaelea lauricola*-inoculated 'Simmonds' avocado stems, respectively, 21 and 42 days after inoculation (dai). Note absence of tyloses in (A); tyloses (*t*) in and smooth deposits (sd) lining vessel walls in (B); and tyloses (*t*), irregular granular structures (*g*), and rough deposits (rd) on vessel walls in (C).

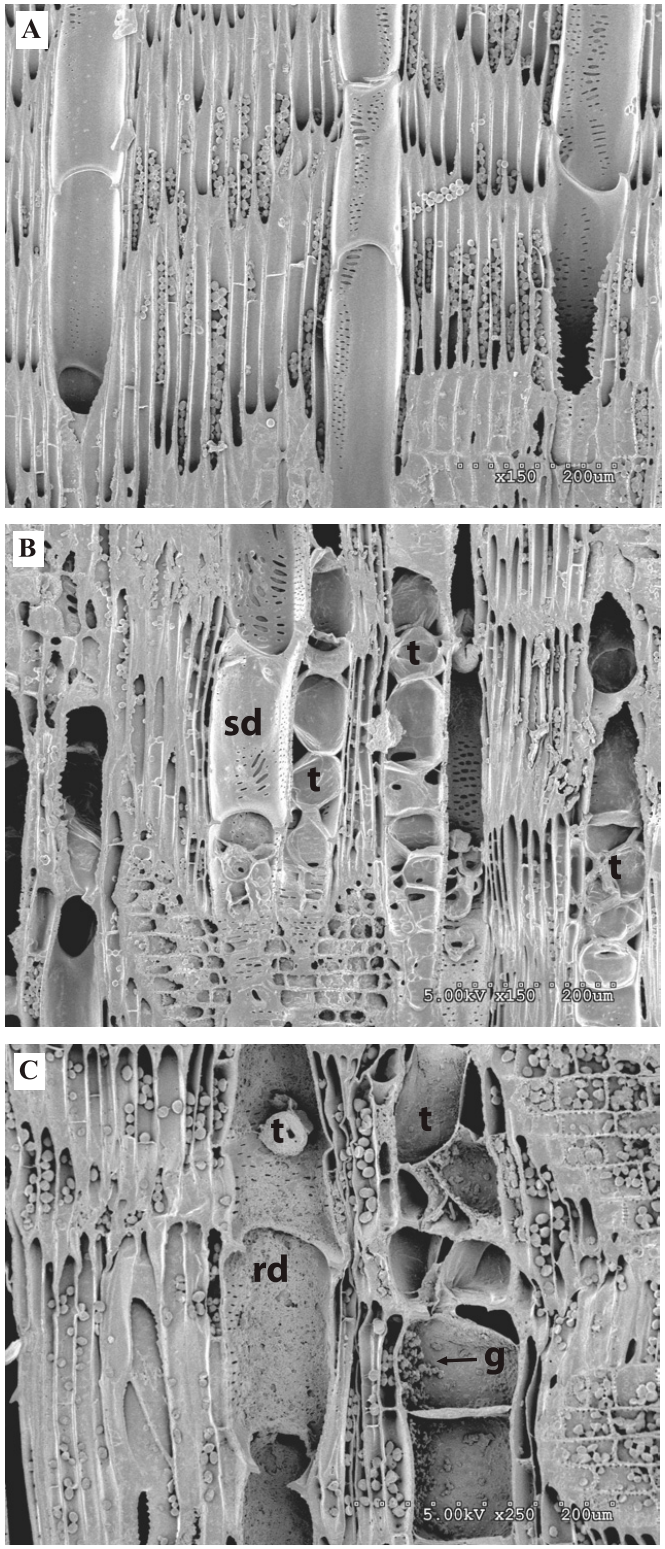
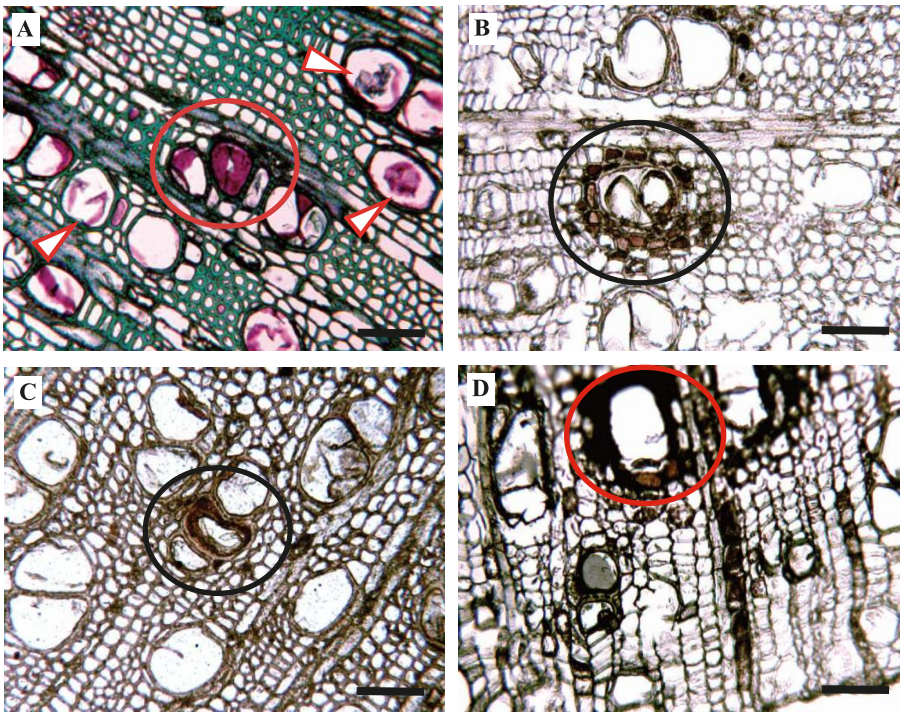


Fig. 5. Light micrographs of transverse sections of *Raffaelea lauricola*-inoculated 'Simmonds' avocado stems at 21 days after inoculation (dai), stained with: (A) toluidine blue O, indicating phenolic and pectic substances in lumena; (B and C) ruthenium red, indicating pectin accumulation; and (D) Sudan III, indicating lipid deposition. Scale bars = 50 μm.



dai, 39.6% and 51.4% of the xylem vessels, respectively, contained tyloses (Table 1). Tylose percentages, dai, and external and internal disease severity were each positively and significantly correlated ($P \leq 0.0001$) (Table 2).

Vessel walls in mock-inoculated plants were smooth and free of coatings and deposits (Fig. 4A). In inoculated plants, smooth deposits were observed on xylem vessel walls by 21 dai (Fig. 4B), and by 42 dai a rough coating had developed on vessel walls with granular deposits of irregular sizes visible in some sections (Fig. 4C). Histochemical staining indicated that these materials were comprised of phenolic compounds, as evidenced by green-colored areas in sections that were stained with toluidine blue O (Fig. 5A), and pectic compounds, as evidenced by pink-colored areas in sections that were stained with ruthenium red (Figs. 5B and 5C). Lipids lined the lumen of xylem vessels in fresh sections that were stained with Sudan III (Fig. 5D).

Mycelia and conidia of *R. lauricola* were associated with xylem vessels and fibers, but in relatively low abundance (Figs. 6A and 6B). Visualization of the pathogen was enhanced in F-WGA stained sections. Although fungal material was not observed in mock-inoculated control plants (Fig. 7A) or inoculated plants at 3 dai (Fig. 7B), it was observed in xylem vessels at 7 dai (Fig. 7C), prior to the development of internal or external symptoms. Colonization and abundance of fungal material increased in xylem vessels, fibers, and adjacent parenchyma after 7 dai (Figs. 7C–7F, Figs. 8A–8C), but by 42 dai began to decrease (Fig. 7F).

Discussion

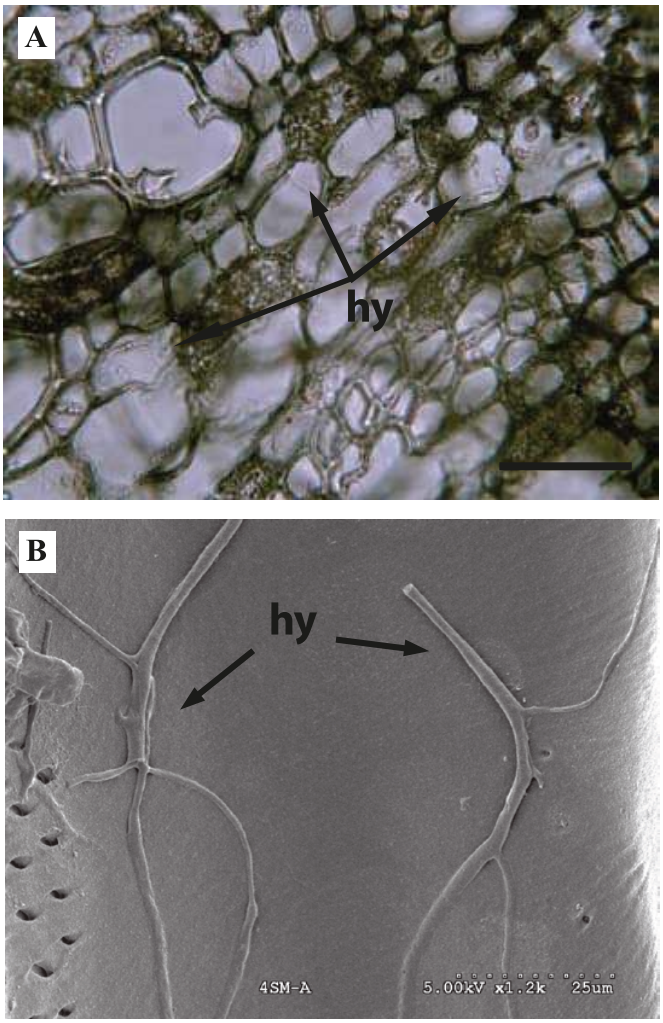
'Simmonds' avocado produces gels and tyloses when infected by *R. lauricola*. This response is typical of those induced by other vascular wilt pathogens of trees (Beckman 1964; Takai and Hiratsuka 1984; Jacobi and MacDonald 1980; Rioux and Ouellette 1991). In the present study, a significant increase in tyloses and gels in xylem vessels 14 dai was associated with the onset of wilting and internal symptom development. Severe wilting (external symptoms >5) was observed when tyloses were observed in greater than 50% of the xylem vessels. Jacobi and MacDonald (1980) reported similar responses in oaks that were affected by oak wilt, caused by *C. fagacearum*; they observed in trees that were severely affected by this disease, tyloses developed in over 50%–60% of the xylem vessels.

The mechanism for tylose development is not well understood. Gerry (1914) suggested that a reduction of pressure in the xylem would pull the adjacent parenchyma cell into the lumen. More recently, Rioux et al. (1998) suggested that embolisms triggered tylose formation in the xylem. Although how vascular wilt pathogens would cause embolisms is not clear, Sperry and Tyree (1988) indicated that oxalic acid produced by *Fusarium* pathogens could mediate changes in host pit membranes that would result in embolisms. Additional work is needed to understand how vascular wilt pathogens impact host xylem function.

Tylose formation and reduced xylem function are associated with symptom development in the avocado × *R. lauricola* pathosystem. Inch and Ploetz (2011) demonstrated that xylem function and hydraulic conductivity decreased as laurel wilt severity increased in avocado. By 21 dai, 65% of the xylem was not functional in artificially inoculated trees with mean external and internal disease severities of, respectively, 4.5 and 5.6.

In the present study, *R. lauricola* spread rapidly and sys-

Fig. 6. Hyphal (hy) distribution in xylem vessels and fibers of avocado stems artificially inoculated of *Raffaelea lauricola* at 21 days after inoculation (dai): (A) light micrograph of transverse section with hyphae (hy) in xylem fibers, and (B) scanning electron micrograph of hyphae on xylem vessel wall. Scale bar = 50 μm.



temically in the susceptible cultivar 'Simmonds'. By 3 dai the pathogen had moved to 5 cm above the inoculation point (where tissue was sampled for histological work), and by 7 dai it was isolated from the entire length of the stem, often 1 m above the inoculation point. In contrast *R. quercivora*, an oak wilt pathogen in Japan, causes only localized infections in its host trees; host mortality is associated with multiple attacks by the associated ambrosia beetle vector, *Platypus quercivorus*, rather than systemic vascular movement of the pathogen (Takahashi et al. 2010).

Perforation plate morphology may also influence the rate at which a vascular wilt pathogen moves systemically in a host plant. Avocado vessel cells have a simple perforation plate at one end and a scalariform perforation plate at the other end; presumably, this anatomy would have little impact on the movement of conidia in the transpiration stream. Red oak, *Quercus rubra*, may be an analogous host tree in that *C. fagacearum* spread systemically and rapidly in this susceptible species owing to the unrestricted vascular movement of this pathogen (Jacobi and MacDonald 1980). In contrast,

Fig. 7. Transverse sections of 'Simmonds' avocado stems stained with fluorescein-conjugated wheat germ agglutinin (F-WGA). No hyphae are evident in sections from (A) mock-inoculated and (B) *Raffaelea lauricola*-inoculated plants 3 days after inoculation (dai). However, infrequent hyphae (hy) were visible 7 dai in (C). The abundance of hy and spores (sp) generally increased by (D) 14 dai and (E) 21 dai in fiber and parenchyma, but appeared to decrease thereafter, (F) 42 dai. Scale bars = 50 μm.

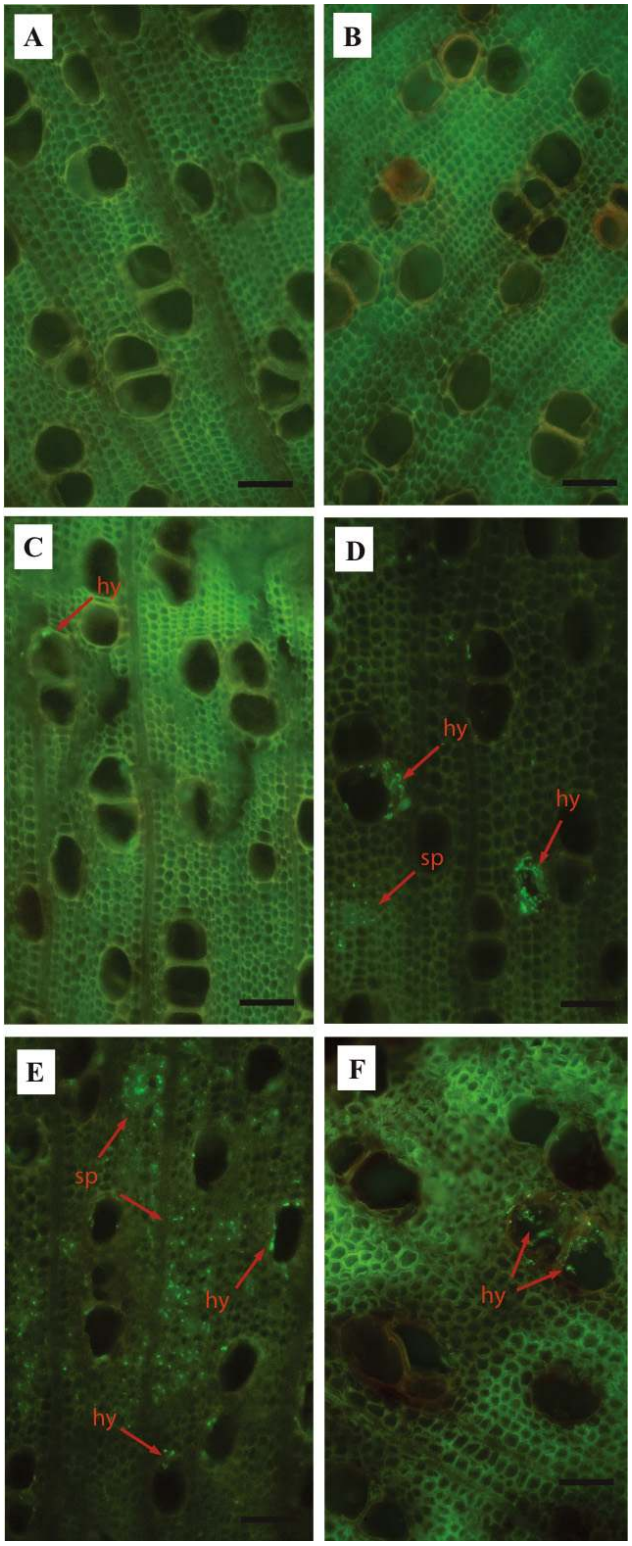
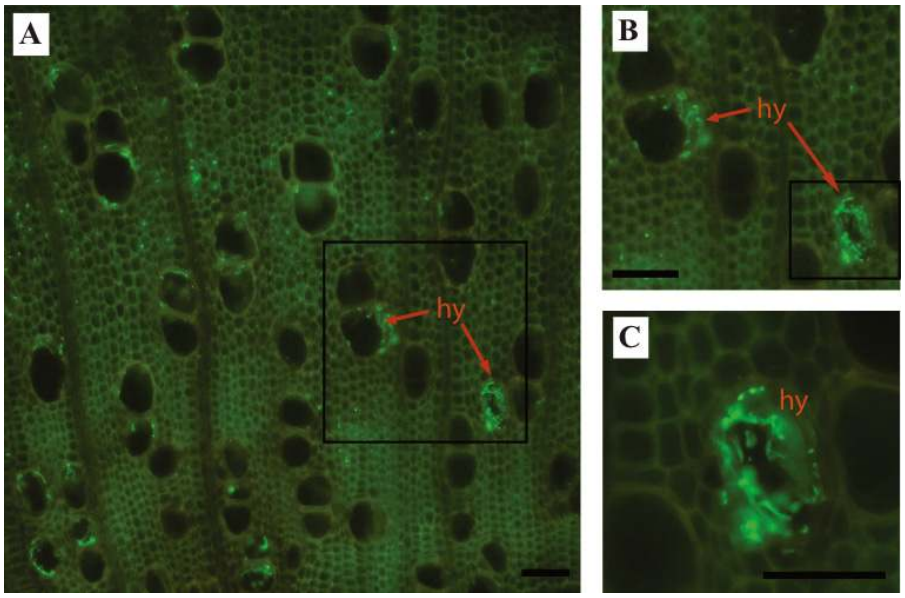


Fig. 8. Hyphal (hy) distribution in *Raffaelea lauricola*-inoculated 'Simmonds' avocado stems. (A) Transverse section stained with fluorescein-conjugated wheat germ agglutinin (F-WGA), and increased magnification, (B) and (C), showing hyphae (hy) within and associated with xylem vessel. Scale bars = 50 μm.



the movement of *C. fagacearum* was restricted in tolerant white oak, *Q. alba*, by perforation plates and the formation of tyloses and gels.

In 'Simmonds', there is surprisingly little histological evidence for the pathogen in host vessels (Ploetz et al. 2011*c* and this study). *Raffaelea lauricola* can grow and multiply in a yeast phase (Harrington et al. 2008). This may account for its rapid movement in plants and its isolation from inoculated plants in which it is scarcely observed. Struckmeyer et al. (1954) did not observe conidia and hyphae of *C. fagacearum* in northern pin oak, *Q. ellipsoidales*, prior to the development of wilt symptoms. In contrast, Jacobi and MacDonald (1980) observed hyphae of *C. fagacearum* 3 dai in their sections, but could not isolate the fungus from tissues until at least 28 dai.

Mycelia and conidia of *R. lauricola* were observed in xylem vessels, fibers and adjacent parenchyma cells of avocado. In general, other vascular wilt fungi tend to be restricted to xylem vessels and the surrounding parenchyma cells (Dimond 1970; Takahashi et al. 2010). Banfield (1968) and Fergus and Wharton (1957) demonstrated that during the initial stages of infection of elm and oak by, respectively, *O. ulmi* and *C. fagacearum*, these pathogens were restricted to the lumen of xylem vessels and spread throughout the plant via transpiration flow. However, after wilt symptoms developed mycelia of these pathogens colonized parenchyma cells. Similar results were observed in *Quercus* spp. infected by *R. quercivora*; mycelia were generally localized in discolored sapwood in vessel elements and ray parenchyma cells (Takahashi et al. 2010).

Conclusion

The rapid colonization and systemic movement of *R. lauricola* in xylem of the culitvar 'Simmonds' suggests that laurel wilt management on this host may be difficult once a plant is infected. The anatomical and histochemical responses of avocado to *R. lauricola* that were observed in the present study are similar to those observed in other vascular wilt diseases of trees, in that conspicuous tylose formation occurs in xylem lumena, and that these structures and phenolic-, pectin-, and lipid-containing gels in the xylem are associated with the development of internal and external symptoms of the disease. All avocado cultivars are susceptible to laurel wilt. Investigations are currently underway; examining the response of other avocado cultivars, with varied levels of susceptibility. Preliminary results indicate that other cultivars respond to infection with the development of tyloses and gels. However, less susceptible cultivars produced fewer tyloses. A better understanding is needed of the temporal and spatial dynamics of this host × pathogen interaction, as it would assist the development of resistant cultivars and may suggest other ways in which this important problem could be addressed.

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