

The effects of *Phytophthora ramorum* infection on hydraulic conductivity and tylosis formation in tanoak sapwood

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Abstract: Tanoak (*Lithocarpus densiflorus* (Hook. and Arn.) Rehder) is highly susceptible to sudden oak death, a disease caused by the oomycete *Phytophthora ramorum* Werres, De Cock & Man in't Veld. Symptoms include a dying crown, bleeding cankers, and, eventually, death of infected trees. The cause of mortality is not well understood, but recent research indicates that water transport is reduced in infected trees. One possible mechanism causing the reduction in hydraulic conductivity is the presence of tyloses in xylem vessels. The development of tyloses was studied in relation to hydraulic conductivity in *P. ramorum*-infected sapwood. Inoculated logs showed a greater abundance of tyloses than noninoculated logs after 4 weeks. Inoculated trees with xylem infections had significantly more tyloses than noninoculated trees. In addition, the increase in number of tyloses was associated with a decrease in specific conductivity, suggesting that tyloses induced by infection with *P. ramorum* may interfere with stem sap flow. Over time, tylosis development increased in tissues farther from the inoculation site, in advance of the vertical spread of infection. The results suggest that infected sapwood contains numerous tyloses, which could significantly impede stem water transport.

Résumé : Le lithocarpe de Californie (*Lithocarpus densiflorus* (Hook. & Arn.) Rehder) est très sensible à l'encre des chênes rouges, une maladie causée par *Phytophthora ramorum* Werres, De Cock & Man in't Veld. Les symptômes incluent le dépérissement de la cime, la présence de chancres suintants et, éventuellement, la mort des arbres infectés. La cause de la mortalité n'est pas bien comprise mais les études récentes indiquent que le transport de l'eau est perturbé chez les arbres infectés. Un des mécanismes qui pourraient réduire la conductivité hydraulique est la présence de thylls dans les vaisseaux du xylème. Dans cette étude, la formation des thylls a été étudiée en relation avec la conductivité hydraulique du bois d'aubier infecté par *P. ramorum*. Après 4 semaines, il y avait plus de thylls dans les billes inoculées que dans les billes non inoculées. Les arbres inoculés dont le xylème était infecté contenaient significativement plus de thylls que les arbres non inoculés. De plus, l'augmentation du nombre de thylls était associée à une diminution de la conductivité spécifique, ce qui laisse croire que les thylls produites en lien avec l'infection de *P. ramorum* pourraient perturber le mouvement de la sève dans la tige. Avec le temps, la formation des thylls a augmenté dans les tissus distants du point d'inoculation, devançant la progression verticale de l'infection. Les résultats indiquent que le bois d'aubier infecté contient une grande quantité de thylls qui pourraient sérieusement nuire au transport de l'eau dans la tige.

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Introduction

In 1994, homeowners in Marin County, California, noticed scattered dead and dying tanoaks (*Lithocarpus densiflorus* (Hook. and Arn.) Rehder) around the Mill Valley area (Svihra 1999). These trees were described as having a dying crown and bleeding bole cankers. By 1999, the disease, called sudden oak death, was described as epidemic on tanoak and coast live oak (*Quercus agrifolia* Née) within a 300 km long area along the coast of central California (Rizzo et al. 2002).

Through completion of Koch's postulates, the pathogen causing mortality of tanoak and coast live oak was shown to be a species of *Phytophthora* (Rizzo et al. 2002). Culture morphology and internal transcribed spacer sequences of DNA matched *Phytophthora ramorum* Werres, De Cock & Man in't Veld, which had previously been recovered only from ornamental plants in Germany and the Netherlands (Werres et al. 2001). *Phytophthora ramorum* is most likely an exotic species that was introduced into California by the international nursery trade and escaped into native forest ecosystems (Rizzo et al. 2005).

Many species of *Phytophthora*, including *P. cinnamomi* (Tainter et al. 2000) and *P. lateralis* (Hansen et al. 2000), have long been known to cause phloem cankers and root rots in trees, and the resulting infections are usually lethal (Tainter and Baker 1996). Symptoms of the diseases caused by these *Phytophthora* species are similar, and usually include cankers containing discolored tissue, and a dying crown. Although infection of phloem and cambium tissue is common, infection of xylem and interruption of water transport have been associated with some root-infecting *Phytophthora* species. For example, when *P. cinnamomi* colonizes

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root tissue of jarrah, *Eucalyptus marginata* Donn ex Smith, in the forests of Western Australia, the pathogen can be recovered from root xylem tissue, and Davison et al. (1994) found evidence of xylem dysfunction and suggested that an interruption of water movement may have contributed to disease symptoms or tree mortality.

Tanoak is highly susceptible to *P. ramorum* and is the host that incurs the highest mortality in mixed evergreen and redwood–tanoak forests of the United States west coast (Rizzo et al. 2005). Symptoms include bole cankers that exude or “bleed” a deep red liquid, and rapid death of the crown. Necrotic lesions, characterized by dark staining in the inner bark, can be observed by removing the outer bark. Disease symptoms have been attributed to the canker girdling the phloem and killing the tree (Davidson et al. 2003), but the exact mechanism by which the pathogen causes mortality is not understood.

Brown and Brasier (2007) demonstrated that *P. ramorum*, *P. kernoviae*, and other *Phytophthora* species could be recovered from the sapwood of some tree species in the United Kingdom. Parke et al. (2007) demonstrated that *P. ramorum* could not only colonize the sapwood of tanoak trees, but also spread through xylem vessels, produce chlamydospores, and reduce sap flow. It is not known how sap flow is reduced when trees are infected with *P. ramorum*, but Parke et al. (2007) reported that vessels occluded by hyphae and tyloses were readily observed in infected tissue.

This research is an attempt to elucidate the mechanism by which *P. ramorum* causes disease symptoms and mortality in tanoak trees. Specifically, we sought to determine the spatial and temporal development of tyloses in tanoak infected with *P. ramorum*, and the relationship between abundance of tyloses and specific conductivity of the xylem. The research was undertaken on both inoculated tanoak logs and inoculated tanoak trees in the field by looking at the time course of tylosis formation and the effects on specific conductivity.

Materials and methods

Log inoculation study

Freshly cut logs are commonly used as models for whole live trees in inoculation studies with *Phytophthora* species, including *P. ramorum* (Brasier and Kirk 2001; Hansen et al. 2005). Healthy tanoak trees were selected from a tanoak stand near Upper Bean Creek, northeast of Brookings, Oregon, and 30 logs, each measuring approximately 45 cm long and 20 cm in diameter, were cut from the trees on 3 October 2005 for this study. Log ends were sealed with wax and logs were transported to Oregon State University. On 5 October, each log was prepared for inoculation by removing a plug of bark to the depth of the cambium at the midpoint along its length, using a 6 mm cork borer. Fifteen logs were inoculated at cambium depth with an agar plug containing *P. ramorum*, while a further 15 were inoculated with a sterile agar plug to serve as wounded controls. The bark plug was reinserted into the inoculation hole and the wound was covered with wet gauze and aluminum foil and sealed with duct tape. All logs were sealed inside 3 mil (0.076 mm thick) plastic sleeves and incubated in a growth chamber at 19–21 °C. At 2, 4, and 7 weeks after inoculation, five inocu-

lated and five wounded control logs were cut into successive 2 cm thick disks with an electric miter saw and processed as follows.

At each inoculation or wound site, a block of wood tissue measuring 1 cm per side and located directly beneath the cambium was removed with a wood chisel. A second block was taken 4 cm from the inoculation site along the axial direction. A third block was taken from the opposite side of the log distal to the inoculation point (Fig. 1) to serve as a noninoculated, unwounded control.

From each of these blocks a smaller block measuring 2–3 mm per side was excised with a straight-edge razor blade and quickly put into FAA (50% ethanol, 5% formalin, 10% acetic acid) for fixing. All samples were subsequently placed under vacuum for 12 h to facilitate fixation and remove air bubbles.

Tissue was prepared for light microscopy by slicing a thin section (approximately 400 µm thick) from each of the fixed blocks by hand with a single-edged razor blade. Each slice was placed on a depression slide with a drop of FAA and a cover slip. Tissue was examined using 100× magnification. The abundance of tyloses in the cross-sectional view of vessels was recorded as the maximum degree (%) to which each vessel was occluded, determined subjectively and rounded to the nearest 10% while focusing through the depth of the tissue sample. Any observable *P. ramorum* structures were also recorded. All vessels in the sample were viewed. The abundance of vessels is relatively low in this species; the average number of vessels viewed per sample was 48.

Field inoculation study

Field-plot establishment

Field plots were established in a Douglas-fir – tanoak stand at El Corte de Madera Preserve in the Midpeninsula Regional Open Space District in San Mateo County, California (N37.41, W122.33, elevation 524–580 m). Boles of understory tanoak trees (7–12 cm DBH) were inoculated on 23 May 2006 with mycelial plugs of *P. ramorum* placed at the cambium. The boles of control trees received agar plugs without the pathogen. The field plot consisted of 20 trees, 11 inoculated and 9 wounded noninoculated controls. Five inoculated and four control trees were harvested on 20 September 2006 by removing a bole section approximately 150 cm long, with the inoculation point in the center. The ends were sealed with wax and the bole sections were wrapped in 3 mil plastic sleeves for transport to Oregon State University, where they were stored at 19–21 °C until processed. The remaining trees (six inoculated and five wounded noninoculated controls) were harvested on 10 July 2007, about 14 months after the experiment was begun.

Sample processing

Bole sections were brought back to the laboratory within 2 days of harvest and processed soon thereafter. Each section was examined for symptoms of infection and photographed with a digital camera. The outer bark was removed and the length and width of inner bark lesions were measured. Sections were cut into 12 cm long subsections with an electric miter saw (Fig. 2a). Subsections A, B, and D were

Fig. 1. Locations on tanoak logs at which tissue samples were taken. Site *a* is 4 cm from the inoculation or wound, site *i* is the point of inoculation or wound, and site *z* is the noninoculated, unwounded site.

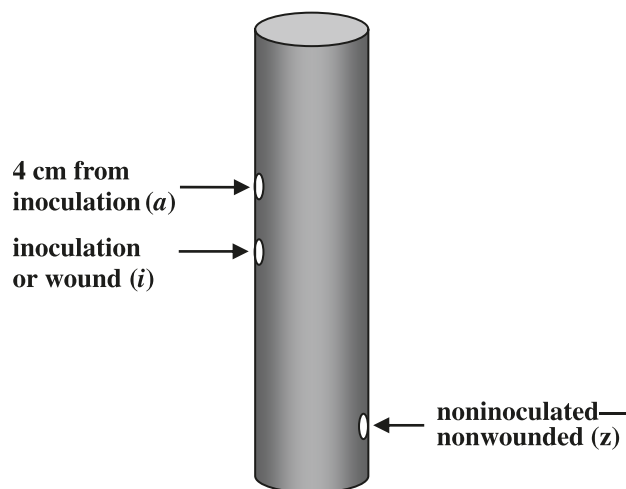
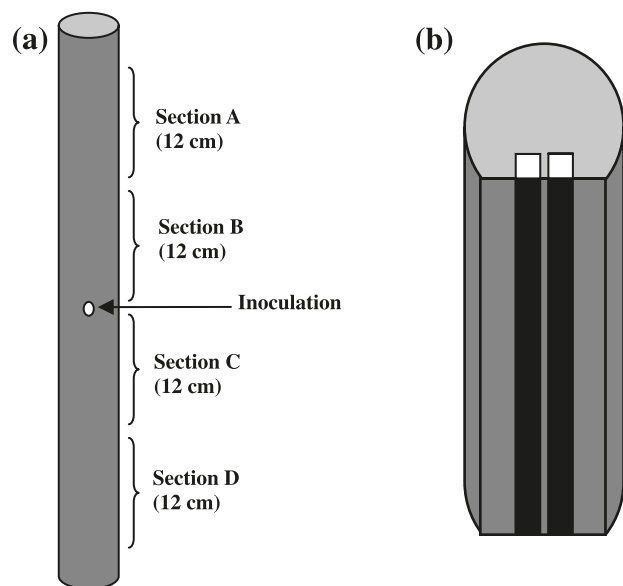


Fig. 2. Diagram of subsections and sticks taken from tanoak logs. (a) Locations of subsections in relation to the inoculation point. (b) The origin of the 1 cm × 1 cm × 2 cm sticks that were removed from the subsections (shaded area).



used for conductivity assays and microscopy, while subsection C was used to confirm the presence of *P. ramorum* using culture and diagnostic polymerase chain reaction (PCR). The vertical extent of sapwood discoloration and the depth of discoloration at the inoculation point were also noted in subsection C. Two sticks each were chiseled from subsections A, B, and D (Fig. 2b) and used for conductivity assays and microscopy.

After excision, each stick was labeled with the sample number and promptly submerged in a 0.01 mol/L HCl solution (pH adjusted to 2) to slow microbial growth. The sticks were then placed under vacuum to remove air bubbles as required for the conductivity assay and stored at 3 °C.

Conductivity assay

Specific conductivity (k_s ; $\text{kg}\cdot\text{m}^{-1}\cdot\text{s}^{-1}\cdot\text{MPa}^{-1}$ (Reid et al. 2005)) describes the permeability of xylem tissue to water for a given length and cross-sectional surface area, and values are calculated according to Darcy's Law as follows:

$$k_s = \frac{Ql}{A\Delta P}$$

where Q is the volume flow rate ($\text{kg}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), l is the length of the stick (m), A is the cross-sectional area of the stick (m^2), and ΔP is the pressure difference from one end of the stick to the other (MPa).

Before conductivity was measured, the ends of each stick were trimmed under water with a razor blade and the dimensions were measured and recorded. Conductivity measurements were made as described in Spicer and Gartner (1998) and Parke et al. (2007). The ends of the sticks were fitted tightly with short segments of vinyl tubing that served as connectors. The sticks were connected on the upstream end to a long vinyl tube filled with a dilute HCl solution (pH 2, filtered to 22 μm) and on the downstream end to the narrow end of a 1 mL pipette. The long vinyl tube, which had a control valve in it, came from an Erlenmeyer flask that was elevated to cause a pressure head in the fluid. Because gravity exerts a force of $0.01 \text{ MPa}\cdot\text{m}^{-1}$ on a column of water, the vertical distance from the top of the solution could be used to calculate the pressure on the upstream end of the sample. Bubbles were removed from the upstream tube and connector.

When the control valve was opened, the fluid passed through the sample and into the pipette. Q was measured by recording the time required for the solution to reach regular volume increments within the pipette. By using lap mode on the stopwatch, up to 10 measurements could be recorded per sample. The average of these measurements was used to calculate k_s . After the conductivity measurements were made, each stick was returned to a dilute HCl (pH 2) storage solution.

Since k_s is dependent on the conductive area of the vessels in the tissue sample, values were normalized by dividing them by the potential conductive area (%) of each sample (determined as described below) and the normalized k_s values were used in comparisons of conductivity. Normalization is particularly important in this radial-porous species because zones of the cross section vary tremendously in abundance of vessels, fibers, rays, and parenchyma.

Microscopic examinations

After conductivity measurements were completed, each stick was fixed in FAA. A sliding microtome was then used to cut thin sections (approximately 30 μm thick) at each end of the stick as well as 1/3 and 2/3 of the way along its length so that a total of four increments were represented. Unstained sections were mounted on glass slides with Polymount (Polysciences, Warrington, Pennsylvania, cat. No. 18606) and covered with a glass cover slip. Three representative digital microscopic images were made of each section using a Zeiss compound microscope (600 $\times$) fitted with a digital camera and imaging software (QCapture Pro 5.0.1, QImaging Corp., Surrey, British Columbia). Assess image analysis software (American Phytopathological Society, St.

Paul, Minnesota) was used to record individual cross-sectional areas of all vessels in the section, as well as the section's total area. We then calculated total percent potential conductive tissue as the sum of the areas of all the vessel lumens in each section, and the hydraulic mean radius, which is the vessel radius that makes the mean contribution to water transport. According to the Hagen–Poiseuille law, the contribution of a single vessel to the total hydraulic conductivity of a sample is proportional to the diameter of that vessel raised to the fourth power (Tyree and Zimmerman 2002). The hydraulic mean radius of the tissue sample is calculated as $\sum r^5 / \sum r^4$, where r is the vessel radius (Kolb and Sperry 1999).

The abundance of tyloses within the vessels was quantified by observing the sample in cross section with a compound microscope under $100\times$ magnification, randomly selecting 100 vessels from each thin section and recording the number containing tyloses.

Statistical analysis

All statistical analyses were performed using S-plus version 6.1 Professional (Insightful Corp., Seattle, Washington). Tables and graphs were constructed using S-plus or Microsoft Excel (Microsoft Corp., Redmond, Washington). Total percent conductive tissue in inoculated sections was compared with that in noninoculated sections with single-factor ANOVA. Individual vessel diameters were calculated from the measured vessel areas, and the frequency distributions of inoculated and noninoculated samples were compared using pairwise t tests on vessel diameters within each size class. The hydraulic mean radii of inoculated and noninoculated samples were compared using single-factor ANOVA.

Tylosis frequencies were analyzed using single-factor ANOVA and performed with arcsine square root tylosis frequencies, since the values were percentages. Specific conductivity measurements were analyzed using single-factor ANOVA performed on nontransformed normalized k_s values. Regression models were constructed on nontransformed tylosis frequencies and normalized k_s values.

The relationship between tylosis frequency and normalized k_s values was analyzed using SAS version 9.1 (SAS Institute Inc., Cary, North Carolina). Initial linear and polynomial regression analyses for harvests 1 and 2, combined and separately, were performed with PROC REG. Regression lines and the final model fit were compared using contrast statements within PROC GLM.

In the field inoculation study, disease failed to develop in several of the inoculated trees. This was evident from the lack of bark-lesion development (external lesion length 0 cm at 4 months, or ≤ 7.5 cm after 14 months) and the small extent of sapwood discoloration in some of the trees. Because the goal of the research was to determine the relationship between *P. ramorum* infection, tylosis development, and specific conductivity, data were analyzed both with and without the trees in which disease failed to develop. These data are designated as either the full set of inoculated and noninoculated trees or the subset of trees. The full data set consisted of all trees listed in Table 1. The total number of samples in the tylosis and conductivity experiments with the full set of trees was 116. This number reflects data taken from 20 trees, three bole subsections per tree, and two sticks

from each subsection, minus four sticks that were lost or damaged. The subset of trees, with five trees excluded, consisted of four inoculated and four noninoculated trees at harvest 1 and two inoculated and five noninoculated trees at harvest 2. Data for the five excluded trees are in italics in Table 1. There were 90 samples (15 trees $\times$ 3 bole subsections per tree $\times$ 2 sticks per subsection) in the tylosis and conductivity experiments for the subset of infected and uninfected trees.

Results

Log inoculation study

Tylosis development

The abundance of tyloses in a cross-sectional view of xylem vessels (hereinafter tylosis frequency) in inoculated logs was compared with that in noninoculated logs over the 7 week period. At 2 weeks there was no significant difference between any of the samples (Fig. 3). At 4 weeks, tylosis frequency was significantly greater in inoculated tissue 4 cm from the inoculation point (site *a*) than at the other sites. Throughout the 7 week period, tylosis frequency increased steadily in noninoculated unwounded tissue (site *z*) and in noninoculated tissue at the wound point (site *w*). At 7 weeks, tylosis frequency decreased significantly in inoculated tissue at both sampling sites (*a* and *i*) and was significantly lower in inoculated tissue at the point of inoculation (site *i*) than at any other site.

Presence of *P. ramorum* structures in xylem vessels

In the log inoculation study, when cross sections of sapwood from the 2 week samples were viewed microscopically, *P. ramorum* hyphae were observed in 10% of the vessels from inoculated tissue at site *a* and 15% of vessels at the point of inoculation, site *i*. No hyphae were observed in wounded or unwounded tissue from noninoculated logs (Fig. 3). The abundance of hyphae decreased between 2 and 4 weeks in both tissue samples taken from inoculated logs (sites *i* and *a*), then remained fairly constant from 4 to 7 weeks. Chlamydospores were observed in samples taken from inoculated logs, most frequently from tissue taken at the point of inoculation at 2 weeks (data not shown).

Field inoculation study

Symptom development

Symptom development in inoculated trees was highly variable (Table 1). Four months after inoculation (harvest 1), external bark lesions ranged in length from 0 to 10.5 cm (mean 7.9 cm) on inoculated trees and from 0 to 1.2 cm (mean 0.9 cm) on noninoculated controls. Fourteen months after inoculation (harvest 2), lesion lengths ranged from 4.5 to 71.3 cm (mean 27 cm) on inoculated trees and from 0 to 1.3 cm (mean 0.5 cm) on noninoculated controls. In the case of inoculated trees, the depth of sapwood discoloration at the point of inoculation averaged 0.6 cm for harvest 1 and 1.3 cm for harvest 2. None of the noninoculated wounded controls exhibited sapwood discoloration. One of the inoculated trees (No. 10206) in harvest 1 remained asymptomatic, and disease developed to only a limited extent in four of the inoculated trees (Nos. 8735, 10129, 10211, 10212) in harvest 2.

Table 1. Summary data for all tanoak trees in the field inoculation study.

Tree No.	External lesion length (cm)	Depth of sapwood discoloration (cm)	Length of sapwood discoloration (cm)	Isolation of <i>P. ramorum</i>	DBH (cm)
Harvest 1 (4 months)					
Inoculated trees					
10128	10.5	0.7	6.6	+	6.8
10133	10.5	0.8	6.4	+	9.3
10205	9.0	0.6	7.6	+	10.0
10208	9.5	1.0	6.0	+	8.0
10206	0.0	0.0	0.0	+	10.2
Noninoculated trees					
10086	0.0	0	0	–	10.0
10087	1.0	0	0	–	8.3
10102	1.2	0	0	–	10.0
10295	1.2	0	0	–	6.3
Harvest 2 (14 months)					
Inoculated trees					
10201	68.3	2.3	9.0	+	6.8
10202	71.3	2.5	90.0	+	12.0
8735	4.8	0.6	9.6	+	11.7
10129	4.5	0.9	6.8	+	9.4
10211	7.5	0.6	7.0	+	8.4
10212	5.6	1.0	0.0	+	6.9
Noninoculated trees					
490	0.0	0	0	–	7.5
10016	0.0	0	0	–	6.5
10104	1.0	0	0	–	10.0
10281	0.0	0	0	–	6.5
10296	1.3	0	0	–	7.3

Note: Data in italics are from inoculated trees in which disease did not develop, or did so only to a limited extent, and that were excluded from some analyses.

Table 2 shows the comparisons of tylosis frequency, specific conductivity, hydraulic mean radius, and external lesion length for all noninoculated and inoculated trees and for the subset of trees showing disease symptoms.

Tyloses

For the full set of trees, tylosis frequency was significantly greater in inoculated trees than in noninoculated wounded trees (Table 2). For all bole subsections in harvest 1 combined, tylosis frequency was 17.4% in the inoculated trees and 0.4% in the noninoculated trees ($P < 0.001$). For harvest 2, these values were 11.2% and 0.4%, respectively ($P < 0.001$) (Table 2). In all subsections from inoculated trees for both harvest dates, except subsection D at harvest 2, tylosis frequency was significantly greater than in corresponding subsections from noninoculated trees. Harvest date affected tylosis frequency ($P = 0.02$), but position in the tree (bole subsection) did not ($P = 0.21$).

Tylosis frequency was 16.3% in the subset of inoculated diseased trees and 0.4% in the noninoculated controls ($P < 0.003$) at harvest 1, with all bole subsections combined (Table 2). At harvest 2 the corresponding values were 29.1% and 0.4%, respectively ($P = 0.001$). Tylosis frequency was influenced by position on the bole ($P = 0.05$) but not by harvest date ($P = 0.12$). Significant interactions occurred between position on the bole and treatment ($P = 0.003$), between position on the bole and harvest date ($P =$

0.017), and between treatment and harvest date ($P = 0.023$). Within the inoculated diseased trees at harvest 1, the greatest tylosis frequency occurred in subsection B, the 12 cm section immediately above the point of inoculation. At harvest 2, the greatest tylosis frequency was observed in subsection A, 12–24 cm above the inoculation point. There was a significant increase in average tylosis frequency from harvest 1 to harvest 2 in subsection A only (ANOVA, $P = 0.018$).

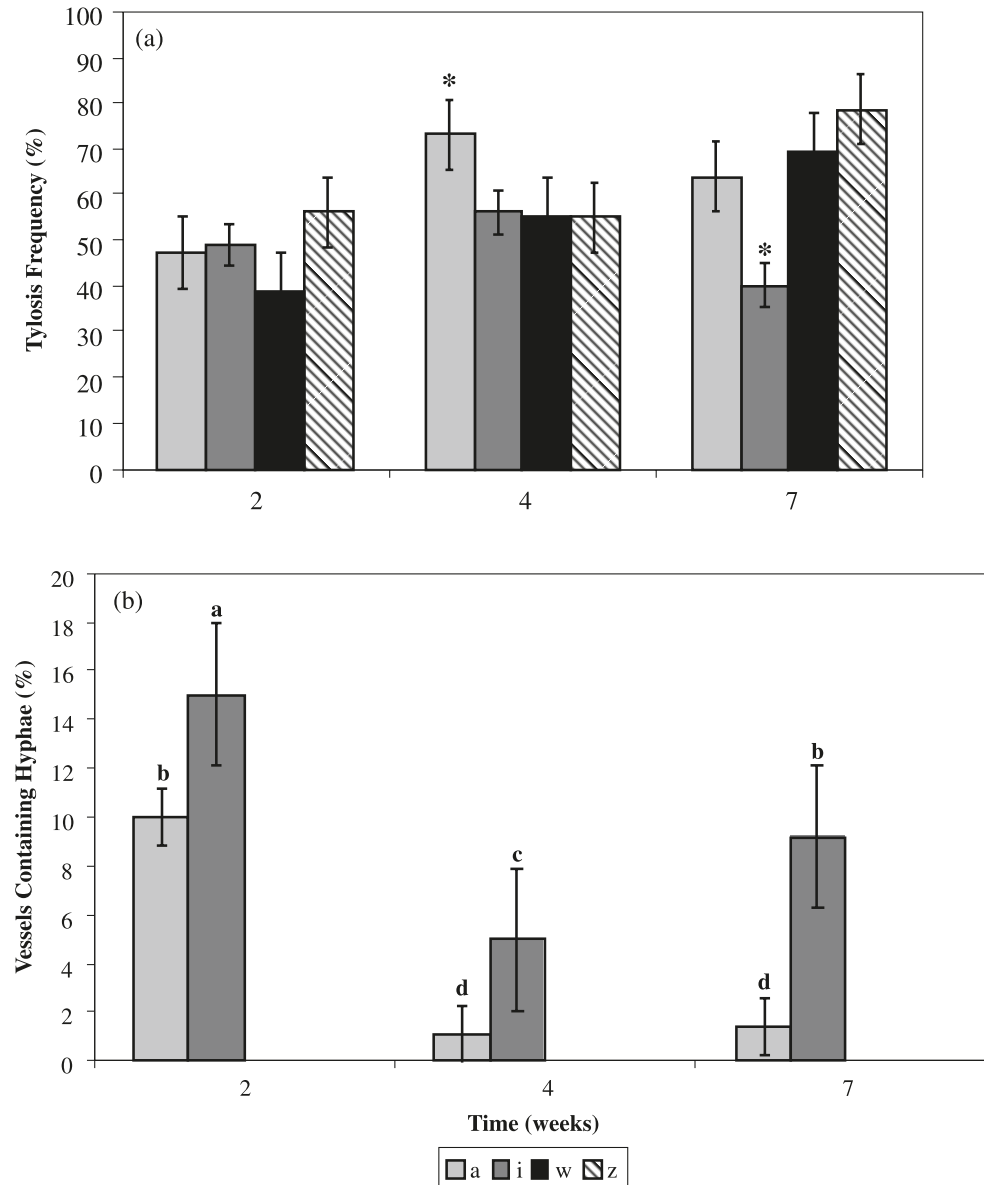
The length of the external bark lesion on the tanoak trees was a fairly good predictor of tylosis development in the xylem vessels for the subset of trees ($P < 0.001$, $R^2 = 0.66$, $n = 15$) (data not shown). There was a significant difference in tylosis frequency between trees that had discoloration in the xylem and those that did not (t test, $P = 0.006$, $n = 15$).

Specific conductivity

For the full set of trees there was a significant difference in normalized k_s values between inoculated trees and noninoculated control trees for all bole sections combined at harvest 1 ($P = 0.03$) (Table 2). By harvest 2, 14 months after inoculation, there was no significant treatment effect on normalized k_s values for combined bole subsections from the full set of trees ($P = 0.65$).

The k_s values were also analyzed after inoculated trees with little or no disease development were excluded. Normalized k_s values for the subset of trees, with all bole sec-

Fig. 3. Tylosis frequencies and occurrence of *P. ramorum* hyphae in vessels in tanoak logs in the log inoculation study; site *i* represents tissue taken at the point of inoculation, site *w* represents tissue taken from noninoculated control logs at the point of wounding, site *a* represents tissue taken 4 cm from the point of inoculation, and site *z* represents unwounded noninoculated tissue taken on the opposite side of the log from the point of inoculation. (a) Tylosis frequencies (mean $\pm$ SE) in a single cross-sectional view of tanoak xylem vessels over time after log inoculations. The asterisks indicate sites, within each time period, where tylosis frequency differed significantly from that at other sites. (b) Abundance of tanoak vessels (mean $\pm$ SE) in a single cross-sectional view containing hyphae. A different letter above the bar denotes a significant difference ($P < 0.05$).



tions combined, were significantly greater for noninoculated trees than for inoculated trees at both harvest 1 ($P = 0.03$) and harvest 2 (ANOVA, $P = 0.006$) (Table 2). The k_s values did not differ significantly ($P > 0.05$) among individual bole subsections at harvest 1, but at harvest 2 there were significant differences in normalized k_s values from inoculated versus control trees for subsections B ($P = 0.04$) and D ($P = 0.02$). For subsection A the difference between treatments was significant ($P = 0.08$) (Table 2). For the subset of diseased trees there was a significant effect of harvest date ($P = 0.008$) on k_s values, but no significant difference among

positions on the bole or treatments ($P > 0.05$). A comparison of normalized k_s values from harvest 1 to harvest 2 was not performed, owing to differences in handling and processing of the samples from the two harvest dates. Two different vacuum chambers were used when removing air from sapwood samples before the conductivity assay was performed, and the difference in the proportion of air that was successfully removed from the sapwood using the two chambers is not known.

To eliminate the possibility that differences in conductivity were due to anatomical differences rather than the host's

Table 2. Comparisons of data for all trees and for the subset of tanoak trees.

		All trees			Subset of trees	
Bole subsection	Harvest	Noninoculated trees	Inoculated trees	<i>P</i>	Diseased trees	<i>P</i>
Tylosis frequency (%)						
Subsection A	1	0.3 (0.4)	6.6 (1.8)	<0.001	6.3 (2.1)	0.03
Subsection B	1	0.5 (0.3)	29.2 (6.4)	<0.001	34.6 (8.6)	0.005
Subsection D	1	0.3 (0.3)	16.4 (7.3)	0.008	7.9 (2.7)	0.03
Combined		0.4 (0.2)	17.4 (3.5)	<0.001	16.3 (4.2)	0.003
Subsection A	2	0 (0)	14.8 (7.2)	0.04	43.3 (13.1)	0.05
Subsection B	2	0 (0)	10.4 (5.3)	0.04	26.5 (11.1)	0.01
Subsection D	2	1.1 (0.6)	7.60 (3.86)	0.10	17.5 (7.5)	0.04
Combined		0.4 (0.2)	11.2 (0.3)	<0.001	29.1 (0.65)	0.001
Specific conductivity, <i>k</i>_s (kg·m⁻¹·s⁻¹·MPa⁻¹)						
Subsection A	1	2.65 (0.43)	2.23 (0.46)	0.51	2.44 (0.63)	0.78
Subsection B	1	2.58 (0.90)	1.41 (0.21)	0.25	1.09 (0.19)	0.15
Subsection D	1	2.44 (0.57)	1.34 (0.28)	0.11	1.24 (0.12)	0.08
Combined		2.57 (0.03)	1.66 (0.20)	0.03	1.59 (0.02)	0.03
Subsection A	2	1.42 (0.45)	1.56 (0.59)	0.85	0.28 (0.07)	0.08
Subsection B	2	2.12 (0.53)	1.37 (0.38)	0.18	0.56 (0.23)	0.04
Subsection D	2	1.71 (0.37)	1.84 (0.50)	0.79	0.70 (0.25)	0.02
Combined		1.75 (0.26)	1.57 (0.28)	0.65	0.51 (0.11)	0.006
Hydraulic mean radius (μm)						
Combined	1	48.8 (1.0)	49.50 (1.24)	0.64	47.2 (1.4)	0.37
Combined	2	49.6 (1.5)	48.59 (2.06)	0.55	50.6 (1.7)	0.64
External lesion length (cm)						
Combined	1	0.9 (0.3)	7.9 (4.0)	0.02	9.9 (0.4)	<0.001
Combined	2	0.5 (0.3)	27.0 (183.5)	0.11	69.8 (1.5)	<0.001

Note: Values are given as the mean with SE in parentheses; *P* values are for *t* tests comparing the full set of inoculated trees with noninoculated trees or the subset of diseased trees with the noninoculated trees.

response to infection, two methods were used: a comparison of vessel size distributions and a comparison of hydraulic mean radii. When vessel size frequency distributions in inoculated and noninoculated tissue were compared, there were no significant differences between treatments for the full set of trees or the subset of trees (data not shown).

A comparison of hydraulic mean radii of the vessels in the three bole sections of inoculated trees with those of the noninoculated controls for both the full set and the subset of trees showed no significant difference between any of the treatments (ANOVA, $P > 0.05$) (Table 2).

Specific conductivity and tylosis development

Regression analysis on the full set of trees indicated that an increase in tylosis frequency was associated with a decrease in specific conductivity for both harvest dates. After log transformation of the *Y* variable to normalize the residuals, a linear regression model gave the best fit to the data for harvests 1 and 2 combined. Addition of X^2 did not improve the fit of the model ($P = 0.6301$). Linear models fit to harvest 1 and harvest 2 data separately gave patterns of residuals that indicated no quadratic component to either regression. The models for harvests 1 and 2 were tested for equality of slopes and intercepts. The slopes were similar ($P = 0.1075$), whereas the intercepts were different ($P = 0.011$). A single model specifying two intercepts and one

slope was fit to the harvest 1 and harvest 2 data. The resulting model, $k_s = 0.7813$ (harvest 1) + 0.2445 (harvest 2) – 0.0243 (tylosis frequency), was significant ($P < 0.0001$, model *df* = 3, error *df* = 112, $R^2 = 0.2396$) (Fig. 4).

The relationship between tylosis frequency and specific conductivity was examined further in the subset of trees that excluded inoculated trees with only limited disease development (Fig. 5). Sapwood from bole subsections with a greater frequency of tyloses within xylem vessels generally showed a decrease in normalized k_s values compared with sapwood from noninoculated control trees. At harvest 1, tylosis frequency was highest in the bole section 0–12 cm above the inoculation site (section B), corresponding to the lowest k_s value. By harvest 2, tylosis frequency was highest and k_s was lowest in bole section A, 12–24 cm above the inoculation site (Fig. 5). For noninoculated wounded controls, tylosis frequency was never more than 1.1% and was associated with higher k_s values.

Discussion

This research shows that infection by *P. ramorum* induces tyloses in the xylem vessels of tanoak stems and demonstrates that tylosis formation is associated with reduced hydraulic conductivity of the sapwood. The development of tyloses has been associated with other host–pathogen sys-

Fig. 4. Relationship of tylosis development to normalized conductivity (k_s) values for the full set of sapwood samples from tanoak trees in the field inoculation study. Linear regression models using the log-transformed Y variable and nontransformed X variable provided the best fit to the data for harvests 1 and 2 combined and for each harvest separately. The slopes for harvests 1 and 2 were similar ($P = 0.1075$) but the intercepts were different ($P = 0.011$). A single model specifying two intercepts and one slope was fit to the harvest 1 and harvest 2 data. The resulting model was $k_s = 0.7813$ (harvest 1) + 0.2445 (harvest 2) – 0.0243 (tylosis frequency) (model $df = 3$, error $df = 112$, $P < 0.0001$, $R^2 = 0.2396$).

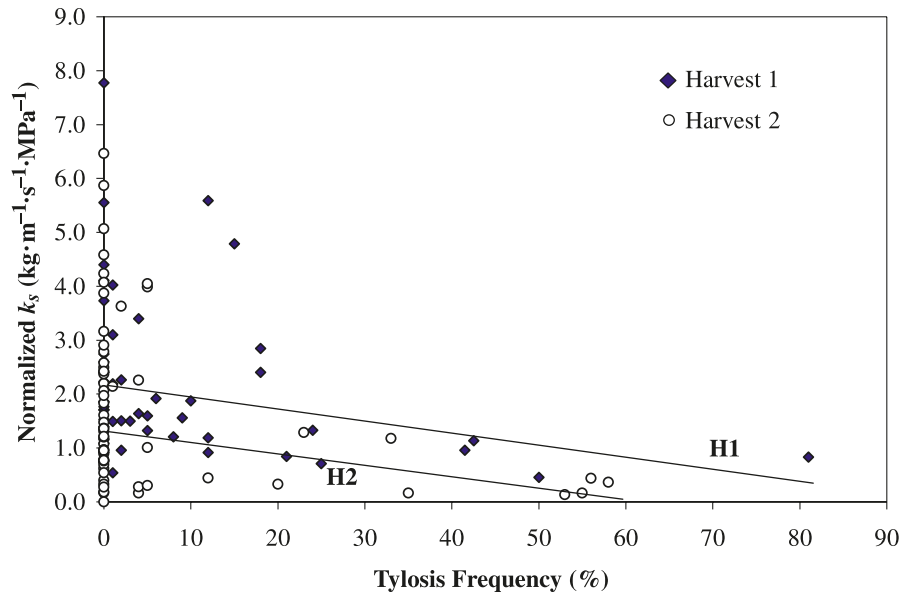
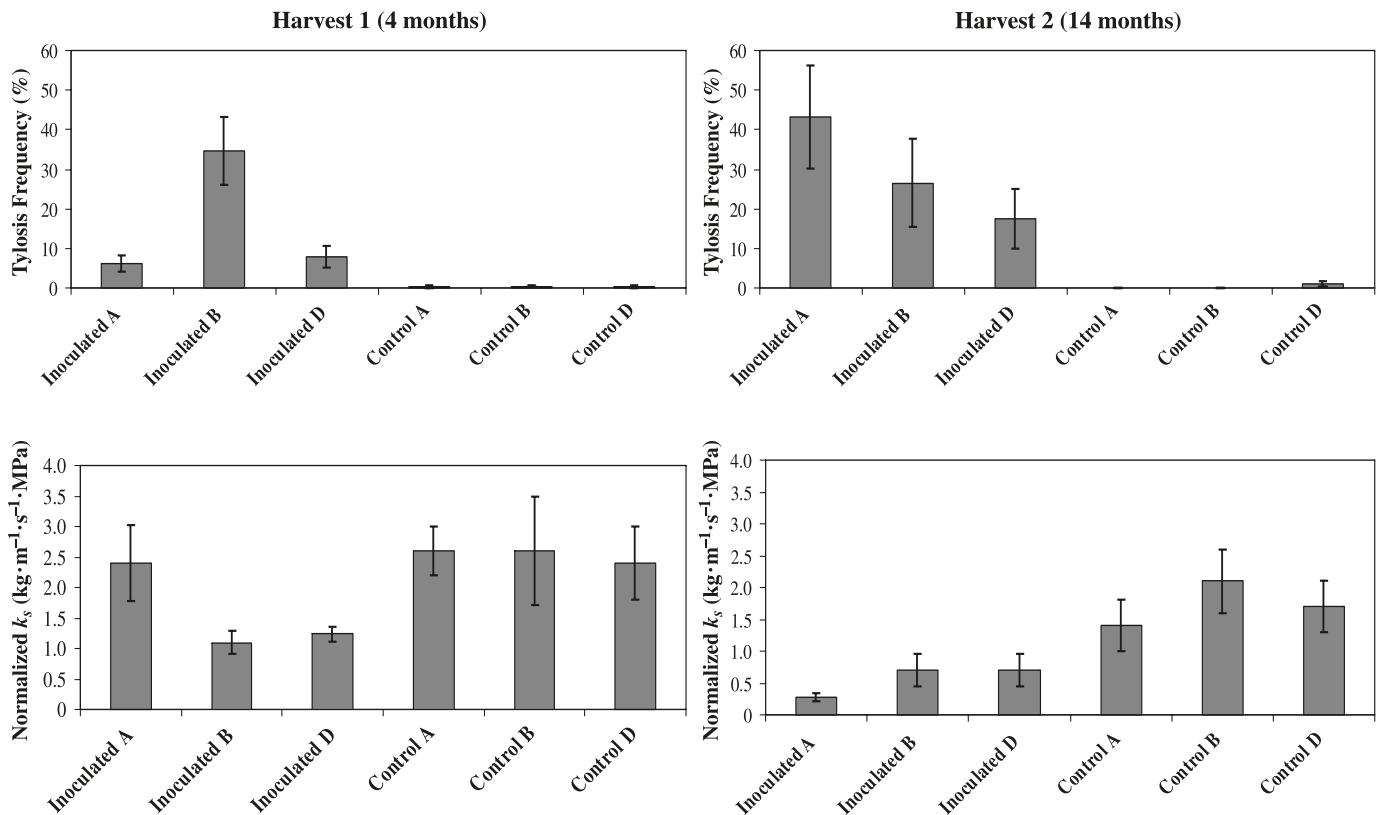


Fig. 5. Tylosis frequency (%) and normalized conductivity (k_s) (mean $\pm$ SE) for the subset of inoculated diseased tanoak trees and noninoculated control trees (bole sections A, B, and D) from the field inoculation study.



tems, such as Dutch elm disease and oak wilt (Beckman 1987), and a resulting loss of conductivity has been reported (Tyree and Zimmerman 2002). These data are consistent

with reduced sap flow and conductivity of tanoak with *P. ramorum* infection (Parke et al. 2007) and offer a mechanism for this reduction.

The specific mechanism by which tyloses are induced has not been studied and is not well understood. According to early hypotheses, a reduction in the internal pressure of the xylem sap pulls the axial parenchyma into the vessel lumen in a ballooning fashion (Gerry 1914). However, contemporary hypotheses implicate air in the vessel lumen (embolisms) as the trigger for active growth of tyloses (Rioux et al. 1998).

The way in which a pathogen causes the embolism required for tylosis formation is also not well understood. Because vessels are transporting water under tension, however, any sufficiently large hole in a vessel wall will allow air, if present, to enter the vessels (Tyree and Zimmerman 2002). Air is apparently quite frequently present in the wood, making up about 26% of the volume of typical hardwood sapwood (Gartner et al. 2004). Beckman (1987) maintains that either the physical damage inflicted on the vessel wall during the infection process, or the ability of a pathogen to fill and physically block the vessel, could allow air to enter the vessel lumen. Parke et al. (2007) reported *P. ramorum* hyphae growing extensively into the vessel lumen, but it is not known whether cell-wall disruption was sufficient to allow air-seeding. Embolisms would, by themselves, restrict sap flow in vivo, but they would have been eliminated by the process of measuring conductivity. It is likely that both embolisms and tyloses contribute to a reduction in stem hydraulic conductivity in vivo.

The ability of volatile molecules such as terpenes and phenolics to reduce the surface tension of water in the xylem sap and induce embolisms has also been reported (Sperry and Tyree 1988). It is not known whether *P. ramorum* produces any of these volatile molecules, but the necrotrophic action of this pathogen could certainly stimulate the production or release of these chemicals by the host (Ockels et al. 2008).

Phytophthora species, including *P. ramorum* (Manter et al. 2007), produce a wide range of effectors — pathogen molecules that influence host cell structure and physiology. These include toxins that contribute to pathogen virulence, and elicitors, considered to be avirulence factors that trigger the hypersensitive response in incompatible hosts. The effects of toxins or elicitors on tylosis formation are not known. *Phytophthora ramorum* can produce effectors that damage host plant cell membranes (Manter et al. 2007). Since tyloses are essentially part of living parenchyma cells, the presence of toxins or elicitors could have the same effect on a tylosis as on other host cells. Furthermore, other *Phytophthora* species have the ability to degrade cell-wall pectin and disrupt the integrity of the cell (Benhamou and Côté 1992). The interaction of the pathogen *P. ramorum* with host tissue has not been well studied, but it is possible that *P. ramorum* uses a similar mechanism, since it is clearly a necrotrophic pathogen.

One disadvantage of the log inoculation experiment was that the destructive process of cutting the tree into logs facilitated the development of embolisms, resulting in the formation of tyloses. Although the inoculated logs showed a significantly higher tylosis frequency than the controls after 4 weeks, the controls became increasingly occluded over time as the cutting process caused vessels to become dys-

functional. Consequently, a long-term investigation of tylosis development during this experiment was not possible.

One surprising result of the log inoculation experiment was the apparent reduction in the number of tyloses over time in the presence of *P. ramorum*. Behind the advancing margin of the infection in inoculated logs, there seemed to be a decrease in tylosis frequency. Noninoculated tissue continued to steadily produce tyloses over time in response to wounding. A similar trend towards a decline in tylosis frequency behind the advancing margin of infection was observed in the field study. One possible explanation is that within the confines of the infected tissue, *P. ramorum* degrades the cell walls or membranes of the newly formed tyloses. Like other *Phytophthora* species, *P. ramorum* requires sterols for growth and sporulation. Manter et al. (2007) postulated that effectors may disrupt sterol-rich host cell membranes to enable the pathogen to better scavenge sterols. The reduction in number of tyloses in colonized tissue over time may result from their breakdown by *P. ramorum*. Tyloses typically appeared thin-walled and hyaline, although a small number of vessels also contained dark-staining, lignified tyloses. Another explanation is that the result is spurious, and that given the variability, a larger sample is needed. It is somewhat hard to imagine that tyloses could be totally degraded and leave no trace on the pit membranes through which they emerged.

The field inoculation study showed that *P. ramorum* can induce the formation of tyloses in living tanoak trees and that the abundance of tyloses is related to a reduction in specific conductivity. In tanoak, the symptoms of sudden oak death resemble the effects of water stress, and this could be at least partially due to the occlusion of the vessels by tyloses. Other host defense responses, including occlusion of vessels by gums or gels, may also contribute to a reduction in hydraulic conductivity, but these were not evident in the present study. Although a more comprehensive study of the effect of *P. ramorum* on the conductive ability of the entire stem is needed, these data show that conductivity is likely to be reduced.

It has been shown that *P. ramorum* hyphae can colonize sapwood vertically via xylem vessels (Parke et al. 2007), and results from this experiment demonstrate that tyloses are formed beyond the advancing hyphae. However, *P. ramorum* hyphae can spread vertically fairly quickly without penetrating the sapwood enough to significantly disrupt sap flow. Consequently, *P. ramorum* may require a significant amount of time to penetrate deeper into the xylem and cause an extensive loss in conductivity.

The vessels of many ring-porous tree species, such as *Quercus alba*, fill with tyloses after just a few years, so the volume of water that is transported up the stem is confined to the vessels of the outer few annual rings (Saitoh et al. 1993). Consequently, infection by a pathogen such as *P. ramorum* that can induce tyloses and reduce conductivity in the xylem vessels in the outer sapwood is likely to have a significant impact on the health of the tree. Conversely, in radial-porous species such as tanoak (Hirose et al. 2005), the xylem vessels in older sapwood are not known to fill with tyloses. It has been shown that the vessels of the latter species are capable of transporting water after more than 20 years and that the volume of water in the stem is con-

stant across its radius (Hirose et al. 2005). It is likely, therefore, that a significant decrease in conductivity across the entire stem of a tanoak tree would require colonization of a large cross-sectional area of the stem.

One complication of this experiment was that the specific conductivity values were highly variable, even among non-inoculated trees. Some sapwood samples had relatively large conductive areas and few tyloses but relatively low specific conductivity. Other samples had a high frequency of tyloses but relatively high specific conductivity. One explanation is that quantifying tylosis frequency does not necessarily describe the degree to which the vessels are occluded, and in some cases, tyloses were too small to have much effect on conductivity, but were still counted. Additionally, microscopic examination involved a very small portion of the stem and may not accurately reflect the abundance of tyloses. It is clear, however, that the formation of tyloses in response to an infection by *P. ramorum* reduces sapwood conductivity. It is important to note that tyloses did not develop as a result of wounding, but only developed in association with an infection.

Based on field examination of infected tanoak trees, greater disease development and tylosis formation in inoculated trees were expected. Rizzo et al. (2002) reported the formation of large cankers and mortality within 2 years after inoculation. They reported an average lesion length of 41.1–42.3 cm 4 months after inoculation compared with 7.9 cm for the same time period in the present study. Trees selected for this study were understory trees that were slow-growing. In addition, weather data indicate that the summer of 2006 was dry, so perhaps moisture was not adequate for extensive lesion development. Despite the small increase in tylosis frequency over time in most of the diseased trees, the results from this experiment show that tylosis development increases vertically with the advancing infection, suggesting that advanced lesions may be associated with extensive tylosis development and a significant reduction in conductivity throughout the sapwood.

This research raises many questions about the spatial and temporal effects of *Phytophthora* spp. infections on the water relations of host trees. The results suggest that after infection, there is an increase in tylosis frequency in the vessels, and also a decrease in specific conductivity, presumably because of the tyloses and the embolisms that triggered their development. Therefore, although we have not shown a link between decreased conductivity and tree mortality, we obtained results that are consistent with the hypothesis that the mortality of tanoak trees caused by *P. ramorum* involves mechanisms similar to the wilting mechanisms observed in Dutch elm disease and oak wilt. Investigations that begin with inoculation and end with tree death would provide valuable information regarding the ability of tanoak to limit the spread of infection and determine whether a loss in conductivity can be extensive enough to cause tree death.

There is now a need to resolve these questions at the whole-tree level and determine how and why different host species exhibit different symptoms. Understanding the mechanism by which *P. ramorum* causes mortality in tanoak and necrosis in other host plants is important for more accurately predicting the circumstances under which an infected tree will die. Accurate predictions of this nature can influ-

ence control or eradication measures used in both forest ecosystems and residential properties.

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