

# *Phytophthora ramorum* Colonizes Tanoak Xylem and Is Associated with Reduced Stem Water Transport

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Accepted for publication 20 July 2007.

## ABSTRACT

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

Isolation, detection with diagnostic polymerase chain reaction (PCR), and microscopy demonstrated the presence of *Phytophthora ramorum* in the sapwood of mature, naturally infected tanoak (*Lithocarpus densiflorus*) trees. The pathogen was strongly associated with discolored sapwood ( $P < 0.001$ ), and was recovered or detected from 83% of discolored sapwood tissue samples. Hyphae were abundant in the xylem vessels, ray parenchyma, and fiber tracheids. Chlamydospores were observed in the vessels. Studies of log inoculation indicated that *P. ramorum* readily colonized sapwood from inoculum placed in the bark, cambium, or sapwood. After 8 weeks, radial spread of *P. ramorum* in sapwood aver-

aged 3.0 to 3.3 cm and axial spread averaged 12.4 to 18.8 cm. A field study was conducted to determine if trees with infected xylem had reduced sap flux and reduced specific conductivity relative to non-infected control trees. Sap flux was monitored with heat-diffusion sensors and tissue samples near the sensors were subsequently tested for *P. ramorum*. Adjacent wood sections were excised and specific conductivity measured. Both midday sap flux and specific conductivity were significantly reduced in infected trees versus noninfected control trees. Vessel diameter distributions did not differ significantly among the two treatments, but tyloses were more abundant in infected than in non-infected trees. Implications for pathogenesis, symptomology, and epidemiology are discussed.

*Additional keywords:* embolism, host water relations, sudden oak death, vascular disease.

The oomycete *Phytophthora ramorum* Werres, Cock, and Man in't Veld causes sudden oak death on certain members of the Fagaceae and foliar blight, and dieback on many forest and nursery species. The list of hosts and associated plants now totals more than 100 taxa (26). This pathogen has killed more than one million trees in coastal California woodlands in the last decade (D. Rizzo, *personal communication*). The pathogen was also discovered in Oregon in 2001, but disease spread in the forest has likely been slowed by an aggressive effort to eradicate infected hosts (9).

Tanoak (*Lithocarpus densiflorus* [Hook. and Arn] Rehder) is highly susceptible to *P. ramorum* and is the host most readily killed by this pathogen in mixed evergreen and redwood-tanoak forests (22). Symptoms include "bleeding" cankers on the bole and rapid death of the crown. Removal of the outer bark reveals extensive areas of necrotic tissue in the inner bark, and tree death is believed to result from girdling of the phloem (6,21). Rizzo et al. (21) observed discoloration of sapwood underlying bark cankers in tanoak and coast live oak (*Quercus agrifolia*) trees.

Our previous research indicated that *P. ramorum* can colonize the xylem of either foliar-inoculated or root-inoculated rhododendrons (15,18) and the pathogen was occasionally isolated from tanoak sapwood (E. M. Hansen, *unpublished data*). In this work we sought to determine if *P. ramorum* regularly colonizes the secondary xylem of tanoak, and to quantify and visualize the frequency and extent of sapwood colonization. In addition to obser-

vations on naturally infected mature trees, we inoculated logs to follow the development of sapwood colonization. For insights into how the pathogen affects the tree, we conducted a field experiment and wood anatomical studies to determine if the presence of *P. ramorum* in sapwood of naturally infected trees interferes with water transport in the bole. Preliminary reports of this work have been published (19,20). The objectives of this study were to determine (i) if sapwood discoloration in naturally infected tanoak trees is indicative of xylem colonization by *P. ramorum*, (ii) to compare the extent of infection in bark and sapwood of logs inoculated with the pathogen, and (iii) to determine if sap flow and sapwood specific conductivity in stems of naturally infected trees is reduced as compared to healthy trees.

## MATERIALS AND METHODS

**Isolation, detection, and microscopy of *P. ramorum* from naturally-infected tanoak trees.** In April 2005, a 40- to 60-year-old mature tanoak (Tree 9135, Oregon Department of Forestry) with a dying crown was detected in an aerial survey of Curry Co., Oregon. Ground checking confirmed the presence of several bleeding cankers on the bole, and *P. ramorum* was isolated from the inner bark. The tree was cut down and a section of the bole (approximately 1 m long) was transported to Oregon State University where it was split longitudinally into quarters. In addition to dark discoloration of the bark associated with cankers, the sapwood had a mottled appearance. For one quarter of the bole section, a miter saw was used to make 42 cross-sectional slices, each approximately 2-cm thick. The end slices were discarded and the remaining slices were labeled in sequence and photographed with a digital camera. Three small chunks (2 × 2 mm) of

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doi:10.1094/PHYTO-97-12-1558

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tissue were removed from 11 randomly chosen slices. The tissue samples were removed from specific locations on the slices as recorded on the photographs. All external surfaces potentially contaminated by the saw blade were trimmed away with a sterile razor blade. Symptoms of wood or bark discoloration at each sample location were noted.

Each resulting block of trimmed tissue (approximately 3 × 3 × 3 mm) was further subdivided for isolation, microscopy, and diagnostic PCR. Tissue for isolation was plated onto *Phytophthora*-selective media (PAR) consisting of 20 mg of pimaricin, 200 mg of sodium ampicillin, and 10 mg of rifampicin per liter added to commercial corn meal agar. Plates were incubated at room temperature (19 to 21°C) and checked every 3 to 4 days for up to 3 weeks for growth of *P. ramorum*. *P. ramorum* was recognized by its distinctive colony morphology, characteristic chlamydospores, and caducous sporangia (27). Tissue pieces for light or scanning electron microscopy were placed in individual vials containing 2% formalin-acetic acid (FAA) (50 ml of 95% ethanol, 5 ml of glacial acetic acid, 10 ml of formalin [37% formaldehyde], and 35 ml of distilled water), placed under vacuum overnight, and refrigerated. The dehydration of samples was carried out in a graded ethanol series (30, 50, 75, and 100%) for 1 h each, and the last step was repeated. For light microscopy, tissue was embedded in plastic using the Technovit 7100 embedding kit following the manufacturer's instructions (EBS Science Co., Agawam, MA). For scanning electron microscopy, the samples were mounted on aluminum stubs, sputter coated with 60/40% by weight of Au/Pd alloy and scanned on an Am Ray 3300 FE model scanning electron microscope. For diagnostic PCR analysis, tissue was finely chopped with a sterile razor blade, placed in a sterile microcentrifuge tube, and frozen at -20°C. DNA extraction and PCR amplification of the internal transcribed spacer (ITS) region of rDNA were carried out using the methods and primers developed by Winton and Hansen (28).

In July 2005, an additional six trees were harvested from a naturally infested stand of approximately 35-year-old tanoak trees in Upper Bean Creek northeast of Brookings, Curry County, Oregon (latitude 42.08826, longitude 124.24914). Tanoak crown symptoms in this stand were first observed in an aerial survey in June 2005 but were not apparent in the February 2005 aerial survey (M. McWilliams, Oregon Department of Forestry, *personal communication*). These trees were part of a physiological study described in a subsequent section. Symptomatic trees had bleeding cankers on the bole, but the crowns were still green, typical of tanoak in the intermediate stages of infection by *P. ramorum*. Asymptomatic trees were of similar size to the symptomatic trees but did not have any obvious bleeding cankers. Twig and foliar symptoms were not observed on any of the trees. Three symptomatic and three asymptomatic trees were felled and cut into bole segments which were labeled to identify their original positions in the trees. Log ends were sealed with wax, the logs enclosed in plastic sleeves, and transported to Oregon State University. Logs were kept at 4°C for 4 to 7 days until further processing.

For each of the logs, outer bark was first removed with a drawknife to reveal necrotic areas in the inner bark. Logs were split into quarters, the inner bark scored to facilitate vertical realignment, and logs were cut with a miter saw into slices 1 to 1.5 cm thick. Consecutive slices were labeled and photographed. From the symptomatic trees, 20 to 40 slices from each of 17 logs were processed. Nine slices were removed from each of the three asymptomatic trees. After cutting, slices were enclosed in plastic bags and stored at 4°C for 2 days before further processing. Previous observations indicated that sapwood discoloration associated with *P. ramorum* infection became more pronounced with this treatment. Slices were examined, and those with discolored sapwood or bark were designated for isolation, PCR detection, and microscopy as described above. In addition, slices adjacent to discolored tissues (up to 1.5 cm away) were sampled

in order to delineate the vertical extent of infection, the depth of penetration into the sapwood, and the spatial relationship of bark infection and sapwood infection. On asymptomatic trees, tissue was similarly sampled from asymptomatic bark and sapwood tissues chosen at random. Occasionally, dark streaking not typical of *P. ramorum* colonization was observed in sapwood tissue from both *P. ramorum* symptomatic and asymptomatic trees. Tissues from these areas were also subjected to plating on selective medium, diagnostic PCR, and microscopy.

Samples from the symptomatic tree felled in April 2005 and the three symptomatic trees felled in July 2005 were combined ( $n = 78$  sapwood samples and 32 bark samples) to analyze the association between discoloration and culture (recovery), detection with PCR, the presence of hyphae or chlamydospores, and the presence of tyloses. Chi-square analysis was used to determine the significance of positive association between each pair of variables. Fisher's exact test was used when  $\geq 25\%$  of the cells in the chi-square analysis had expected counts of less than five. McNemar's exact test was used to determine the significance of disassociation between pairs of variables.

**Tomographic reconstruction.** Digital images from serial slices of bole sections from three infected trees were used to create three-dimensional visual models of discolored sapwood in relation to the external bark cankers. The perimeter of each slice was manually outlined on its image using a stylus and a tablet PC. Margins of the discolored sapwood and bark were also outlined manually after enhancing the contrast in Adobe Photoshop. Slices were digitally aligned and a model generated using the software Reconstruct (8). Tissue samples were removed from every fifth slice for isolation and PCR detection of *P. ramorum* to determine the relationship between tissue discoloration and presence of the pathogen.

**Log inoculation study: Effect of inoculum placement on infection spread.** Healthy-appearing tanoak trees from a non-infested site in Curry County, Oregon were cut down in October. Bole sections 15 to 30 cm in diameter were cut into logs 45 cm long, and log ends were immediately sealed with wax to retard moisture loss. The freshly cut logs were enclosed in plastic sleeves for transportation to Oregon State University where they were randomly assigned to each of four treatments. There were three inoculum placement treatments (in the bark, at the cambium, or in the sapwood). A fourth treatment consisted of a wounded but noninoculated control in which an agar plug without *P. ramorum* was placed 2 cm into the sapwood. There were 10 replicates for each of four inoculation treatments for a total of 40 logs.

At the midpoint of each log, a single 6-mm-diameter hole was made with an electric drill midway into the bark, just to the cambium, or 2 cm into the sapwood. A 5-mm agar plug was removed from the margin of a 2-week-old culture of *P. ramorum* (isolate 2027.1) growing on 1/3 V8 juice agar and inserted with an inoculation "plunger" in such a way that other tissues were not contaminated. The wound was covered with a square of moistened cheesecloth and a 6 × 6 cm square of aluminum foil held in place with duct tape (11). Logs were replaced in plastic sleeves, the sleeves were sealed with tape, and the logs incubated upright in a growth chamber at 19 to 21°C. Eight weeks later, logs were removed from their plastic sleeves, and the external bark was scored in the vicinity of the wound to determine the length and width of the inner bark canker. The log was then cut with a miter saw into 1- to 2-cm-thick cross-sectional slices. Slices were numbered sequentially, their thickness measured, discoloration noted, and photographed. Tissue samples (approximately 3 mm<sup>3</sup>) were removed from both the bark and sapwood to establish the length and depth of the infection by culture and diagnostic PCR. Samples were removed from specific known locations at the axial margin of the bark canker, from the sapwood at the longitudinal margin of discoloration, and in the sapwood inwards from the point of inoculation towards the pith. Samples were removed

from the last discolored tissue and from adjacent nondiscolored tissue in the next slice or 1-cm section. Tissue samples were divided in half and plated onto PAR, or frozen for possible later extraction of DNA and diagnostic PCR analysis. Plates were observed for growth of *P. ramorum* from the tissue samples. The duplicate tissue sample was processed for diagnostic PCR only when *P. ramorum* was not isolated from the plated sample. Data were analyzed to compare the extent of discoloration and recovery/detection of *P. ramorum* in bark versus sapwood samples in logs inoculated at each of three sites. A two-factor hierarchical model was used, with factors tissue (bark or sapwood), inoculation site, tissue–inoculation site, and a random effect of location to account for the pairing of bark and sapwood samples.

**Log inoculation study; time course of infection spread.** A time course experiment was initiated at the same time as the inoculum placement study to observe the development of infection. Logs were obtained and prepared as previously described, but agar plugs with or without *P. ramorum* were placed at a single site at the cambium only, and an increment borer was used for making a hole, rather than the electric drill. There were five replicate logs × two inoculation treatments (inoculated and control) × two time points (4 and 7 weeks after inoculation) for a total of 20 logs. Tissue samples were processed as described previously. Statistical analysis with single-factor analysis of variance (ANOVA) was used to compare the extent of bark and sapwood discoloration and recovery/detection at each of two sampling dates.

**Physiological studies.** The Upper Bean Creek site was also used for a study on sap flux and specific conductivity. The study site was the same naturally infested stand of approximately 35-year-old tanoak trees northeast of Brookings, Curry County, Oregon described previously. Three asymptomatic, noninfected trees with bole diameters similar to three symptomatic infected trees were chosen for comparison (Table 1). Crown size was estimated by measuring crown diameter on two perpendicular transects before the tree was felled as well as measuring total tree height and live crown ratio once the trees were felled. Geometric calculations show that on average the infected trees we studied had larger crowns than the control trees (Table 1).

On 7 July 2005, three heat dissipation sap flow sensors (13) were installed into the sapwood of the boles of each of the six trees. On the symptomatic trees, sensors were placed around the bole either directly above or directly below bleeding cankers (depending on tree morphology). On the control trees, the three sensors were arranged equally spaced around the tree circumference at a similar height to the symptomatic trees. Sensors, constructed as per James et al. (13), consisted of two temperature sensors that were inserted 1.5 cm into the xylem and vertically separated by 10 cm. The two sensors were connected by a copper thermocouple. All sensors were coated with a thermally conductive silicone heat sink compound before installation and were shielded from sunflecks through the canopy with reflective insulation throughout the period of data collection. Signals from the sensors were monitored every minute, and 10-min means were recorded by a data logger (CR10X; Campbell Scientific, Inc., Logan, UT) equipped with a 32-channel multiplexer (AM416; Campbell Scientific, Inc., Logan, UT).

TABLE 1. Characteristics of tanoak trees in the field study

Treatment	Tree	DBH (cm) <sup>z</sup>	Crown volume (m <sup>3</sup> )
Control	4573	22.1	91
Control	4575	26.9	106
Control	4574	18.1	38
Infected	9160	21.1	171
Infected	4572	22.5	71
Infected	9161	21.0	131

<sup>z</sup> Diameter at breast height (taken at 1.37 m above the ground).

Standard methods were used to estimate sap flux ( $J_s$ , g m<sup>-2</sup> s<sup>-1</sup>) from a voltage drop across the thermocouple. For each thermocouple the voltage drop signal represents the relative rapidity of sap flux across a region of wood as a temperature difference between the heated downstream sensor and the unheated upstream sensor. These temperature differences were used to calculate sap flux from the following empirical calibration of Granier (10):  $J_s = 119k^{1.231}$ , where

$$k = \frac{\Delta T_m - \Delta T}{\Delta T}$$

$\Delta T$  is the temperature difference between the two sensors, and  $\Delta T_m$  is the temperature difference when sap flux is assumed to be zero (the maximum temperature difference recorded in a 24-h period). During data analysis, it became apparent that one of the sensors in infected tree 9160 malfunctioned during most of the measurement period and so it was not included in any analyses. Sap flow data were plotted for the 16-h period. Repeated measures ANOVA was conducted for 84 time periods of log-transformed sap flux measurements with the PROC MIXED model (SAS version 8e statistical software, SAS Institute, Cary, NC) taking into account the nonindependence of sensors located within trees. The fixed effects were treatment, time, and treatment–time. Random effects were tree, sensor, and tree–sensor. Because sap flux should not differ between infected and noninfected trees except during periods of peak sap flux, repeated measures ANOVA was also conducted for the highest 50% of non-zero values for sap flux (37 time periods).

Sap flow sensors were removed and trees felled on 12 to 13 July. Entire bole sections 70 to 130 cm long, containing the regions where sensors had been located, were labeled, excised, their ends sealed with wax, wrapped in plastic bags, and transported to Oregon State University where they were refrigerated until further processing on 14 to 15 July. The remainder of the felled trees was carefully examined for symptoms along the entire length of the bole and branches, and their position was noted. A total of 11 additional 30- to 150-cm-long bole sections not containing sap flow sensors were collected from the infected trees in the study. These were transported to the laboratory to determine the presence and location of *P. ramorum* in the tissues by culture and PCR detection.

In the laboratory for each bole section, the outer bark was removed to reveal canker development, if any, in the inner bark. Each log was split longitudinally and then a miter saw was used to remove a 1-cm-thick cross-section (slice) near the top of each sap flow sensor location. To determine if *P. ramorum* was present in the tissue, small samples of sapwood and bark from each of three slices per log were plated onto a selective medium (PAR), frozen for diagnostic PCR, and fixed for scanning electron microscopy and light microscopy as described previously.

For each sap flow sensor, three xylem segments (each approximately 1 × 1 × 10 cm, radial × tangential × longitudinal, respectively) were chiseled from surrounding wood for the measurement of specific conductivity ( $k_s$ , kg m<sup>-1</sup> s<sup>-1</sup>MPa<sup>-1</sup>). Thus, there were 54  $k_s$  samples (6 trees × 3 locations/tree × 3  $k_s$  samples per sensor). Two of the  $k_s$  segments were located on either tangential side of the sap flow sensor and the third was located just inside, radially from the sensor. Specific conductivity is an expression of how permeable a segment of xylem is for a given length and cross-sectional area, and is calculated according to Darcy's law as

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

where  $Q$  is the volume flow rate (m<sup>3</sup>s<sup>-1</sup>),  $l$  is the length of the segment (m),  $A$  is the cross-sectional area of the segment (m<sup>2</sup>), and  $\Delta P$  is the pressure difference across the segment (MPa).

After segments were removed from the wood, they were stored for up to a week before measurement of  $k_s$ . To inhibit microbial

growth during storage, segments were stored at 3°C and submerged in a dilute solution of HCl (pH 2, filtered through a 22- $\mu\text{m}$  Millipore filter). Immediately before measuring conductivity, the ends of each segment were recut under water and the dimensions of the segment were measured with calipers. Clear latex tubing was slipped over each of the segment's ends, and then the entire segment was placed inside the pressure-sleeve apparatus (24), which holds a thin latex sleeve against the sample to prevent water from being pushed out from the sample's sides. The tubes extended beyond the pressure-sleeve apparatus. For the measurement of  $k_s$ , HCl solution (pH 2, filtered to 22  $\mu\text{m}$ ) was pushed through the tubing with a pressure head of approximately 0.006 MPa. Volume flow rate,  $Q$ , was estimated as the average of three readings per sapwood segment of the time it took fluid to pass at least six successive marks on a 1-ml pipette that was attached to the downstream latex tube. For all  $k_s$  measurements the temperature of the solution was kept near 20°C. Data were analyzed with the PROC MIXED model (SAS version 8e statistical software, SAS Institute), with random effects assigned to xylem position, tree, and xylem position-tree to account for the nonindependence of samples.

Vessels that contain air blockages (embolisms) cannot transport water. Drought-induced embolisms are caused by air-seeding, in which a small bubble is pulled through a pore in the pit membrane in the vessel and, because the water column is in tension, the bubble expands quickly to fill the entire vessel (25). The higher the water tension (the more negative the water potential), the smaller the hole through which a bubble can be pulled. Therefore, we estimated the mean maximum water tension gradients ( $\text{MPa}^{-1} \text{m}^{-1}$ ) in the trees. The gradient is estimated as the ratio of the maximum  $J_s$  (the water volume passing a cross-sectional area per unit time) to  $k_s$  (the water volume passing a cross-sectional area per unit time and pressure gradient). The  $k_s$  values were taken as the mean of the three  $k_s$  values determined near each sensor.

To determine if differences in  $k_s$  resulted from differences in the proportion of cross-sectional area occupied by vessels or the diameters of xylem vessels, transverse microtome sections were made from each sample for which we had measured specific conductivity ( $n = 54$ ). The proportion of cross-sectional area occupied by vessels was based on one field of view at  $\times 60$  magnification for each of the 54 slides using image analysis software (Assess, APS Press, St. Paul, MN). Data were analyzed with single-factor ANOVA.

A subset of slides was analyzed for vessel diameter distributions, selecting one slide each from inner and outer xylem for 16 of the 18 sensor locations. Slides from two of the samples (one infected and one control) became desiccated during storage and were not used. The slides were digitally photographed, and the maximum and minimum width of 50 vessels per slide were averaged ( $n = 800$  per treatment) to estimate vessel diameter. A frequency distribution of vessel diameters assigned to 20  $\mu\text{m}$  size classes was plotted for control versus infected trees. Mean vessel diameter (which tells the average size of vessels) and mean hydraulic vessel diameter (which is a weighted average of vessel size depending on how much flow it contributes [14]) of infected and control treatments were compared with  $t$  tests. This was done to determine if inherent vessel characteristics in individual trees would explain the observed differences in sap flow and specific conductivity between infected and control trees. Because the flow through vessels increases with the fourth power of the diameter, the larger vessels contribute more to flow than do the smaller vessels (25). If all vessels in the sample had the diameter represented by the mean hydraulic vessel diameter, they would all contribute equally to the flow. Mean hydraulic vessel diameter is calculated as  $\Sigma d^5/d^4$ . Lastly, the microscopic slides were examined for the frequency of tyloses, structures similar to primary cell wall that are extruded from parenchyma cells into the vessels post-cambially. One slide from each sample was examined in the

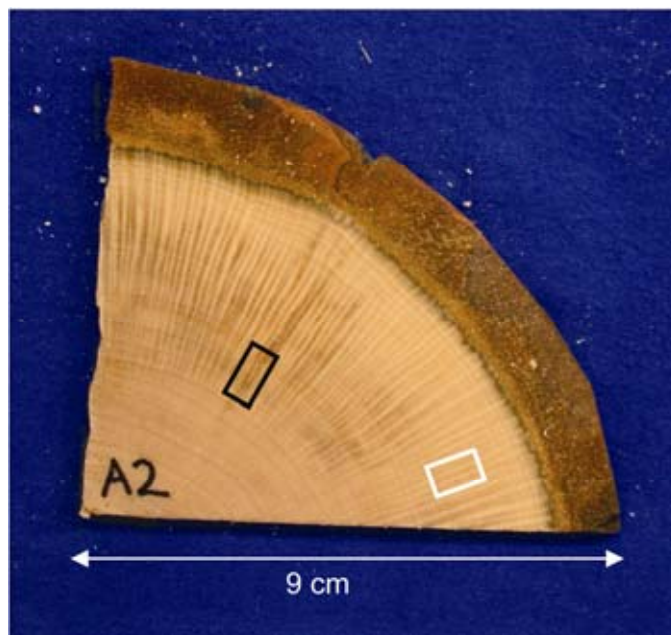
$k_s$  study ( $n = 54$ ) under the compound microscope ( $\times 100$ ), scoring each of 100 vessels per slide for the presence or absence of tyloses. Data on mean vessel diameter, mean hydraulic vessel diameter, and frequency of tyloses were analyzed with single-factor ANOVA.

## RESULTS

**Isolation, detection, and microscopy of *P. ramorum* from naturally infected tanoak.** *P. ramorum* was found in each of the four symptomatic trees but in none of the three trees that lacked bleeding cankers. Bark symptoms were typical of those described for *P. ramorum* on tanoak (21) with one to several bleeding cankers per tree, and underlying necrotic lesions in the inner bark. In addition to showing these bark symptoms, the sapwood of these trees often appeared mottled in longitudinal view (apparent in the split logs). In cross section, this appeared as grayish discoloration of the sapwood, up to 4 cm from the cambium (Fig. 1). Discolored areas occurred in radial patches adjacent to healthy-appearing white or light brown sapwood. Under a dissecting microscope, the grayish discoloration appeared to be associated with the ray cells and the tissues immediately surrounding them. Sapwood from the three asymptomatic trees (without bleeding cankers) was not mottled or discolored, and appeared white to light brown.

*P. ramorum* was isolated (cultured) and/or detected (with diagnostic PCR) from 83% of discolored sapwood samples (Table 2). Chi-square analysis indicated that discoloration was strongly associated with *P. ramorum* isolation ( $P < 0.0001$ ) and with PCR detection ( $P = 0.0002$ ) (Table 3). *P. ramorum* was also detected by PCR in 23% of adjacent nondiscolored sapwood, indicating a significant dissociation with discoloration (McNemar's exact test,  $P = 0.02$ ). Not surprisingly, culture and PCR detection were strongly associated ( $P < 0.0001$ ). *P. ramorum* was not recovered or detected from 60% of nondiscolored sapwood samples in infected trees.

*P. ramorum* was recovered (cultured) and/or detected (with diagnostic PCR) from 84% of discolored bark samples (Table 2).



**Fig. 1.** Grayish discoloration of ray cells in tanoak sapwood from tree 4572 characteristic of infection by *Phytophthora ramorum*. *P. ramorum* was isolated from the location marked with a black rectangle 4 cm from the cambium, but was not isolated from the nondiscolored location marked with a white rectangle 2 cm from the cambium. Note that discolored sapwood is not contiguous with discolored cambium or bark lesions.

For discolored bark samples, PCR resulted in detection of *P. ramorum* in an additional 32% of samples as compared to those which yielded the pathogen in culture. It was recovered or detected at much lower frequency (43%) from adjacent non-discolored bark tissue in infected trees. For bark, discoloration was not as good a predictor of *P. ramorum* isolation ( $P = 0.10$ ) as it was for sapwood; the association of bark discoloration with PCR detection was stronger ( $P = 0.05$ ).

Pathogen structures were observed in 11 of 20 samples that were PCR- and culture-positive for *P. ramorum*, but were not observed in any of the 23 samples of nondiscolored sapwood that were PCR- and culture-negative (Table 2). Sapwood discoloration and recovery on PAR were good indicators of the presence of hyphae and chlamydo spores in the tissues ( $P = 0.002$ ). Interestingly, in tissues positive for *P. ramorum*, pathogen structures were encountered more frequently in the nondiscolored areas of sapwood (18 of 19 samples) than in adjacent discolored sapwood (12 of 22 samples), suggesting that the pathogen structures may precede discoloration of the tissues. Hyphae were commonly found within the lumens of vessels and fiber tracheids near vessels, and within ray parenchyma cells (Fig. 2). Terminal and intercalary chlamydo spores consistent with the species description for *P. ramorum* were also observed in xylem vessels (Fig. 2F). Ray parenchyma cells adjacent to infected tissues were filled

with a dark, opaque material. Hyphae were commonly found in bark (Fig. 2D). No hyphae or chlamydo spores were observed in sapwood (Fig. 2A) or bark samples from the control trees.

Tyloses were observed within vessels of both infected and control trees, but appeared to be more abundant in infected trees (Fig. 2B and H). Although not quantified in this experiment, tyloses were quantified in the physiological study. In *P. ramorum*-infected trees, tyloses were so large and abundant that vessels often appeared to be completely occluded. The presence of tyloses in sapwood tissue from infected trees was strongly dissociated with discoloration, culture on PAR, and detection with PCR (McNemar's exact test,  $P < 0.001$ ) (Table 3) indicating that tyloses form some distance away from the infection and may be degraded by the pathogen.

**Tomographic reconstruction.** Virtual reconstruction of symptomatic trees enabled visualization of the pattern of infection (Fig. 3). Vertical alignment of the discolored areas within the sapwood, and confirmation of *P. ramorum* presence in many of these tissues, suggests the possibility of axial spread within vessels as well as radial spread through ray parenchyma from bark cankers into sapwood (or from sapwood to bark). The discolored areas in the sapwood were sometimes discontinuous with discolored areas of the cambium and bark. Xylem discoloration occasionally extended beyond bark necrosis.

TABLE 2. Relationship between discoloration, recovery and/or detection of *Phytophthora ramorum*, and presence of pathogen structures in tissue samples from mature naturally infected tanoak trees

Tissue	Recovery <sup>x</sup>	Detection <sup>y</sup>	Number of samples (%)	Chlamydo spores or hyphae present
Sapwood, nondiscolored (n = 48)	–	–	29 (60.4%)	0/23
	–	+	14 (29.2%)	14/14
	+	–	1 (2.1%)	0/1
	+	+	4 (8.3%)	4/4
Sapwood, discolored (n = 30)	–	–	5 (16.7%)	0/2
	–	+	1 (3.3%)	0/1
	+	–	1 (3.3%)	1/1
	+	+	23 (76.7%)	11/20
Bark, nondiscolored (n = 7)	–	–	4 (57.1%)	0/1
	–	+	2 (28.5%)	nd <sup>z</sup>
	+	–	0 (0.0%)	
	+	+	1 (14.3%)	0/1
Bark, discolored (n = 25)	–	–	4 (16.0%)	nd
	–	+	8 (32.0%)	4/6
	+	–	0 (0.0%)	
	+	+	13 (52.0%)	5/5

<sup>x</sup> Determined by culturing on semiselective medium.

<sup>y</sup> Determined by diagnostic polymerase chain reaction.

<sup>z</sup> nd = not determined.

TABLE 3. Statistical significance of the association between discoloration, culture of *Phytophthora ramorum*, polymerase chain reaction (PCR) detection of *P. ramorum*, presence of fungal structures,<sup>y</sup> and tyloses

Tissue	Association	P values	
		Chi-square analysis <sup>z</sup>	McNemar's exact test
Sapwood (n = 78)	Discoloration–culture	<0.0001	1
	Discoloration–PCR	0.0002	0.02
	Culture–PCR	<0.0001	0.002
	Discoloration–fungal structure	0.002	1
	Fungal structure–culture	0.0002	1
	Fungal structure–PCR	0.07	0.05
	Discoloration–tyloses	0.2	<0.0001
	Tyloses–culture	0.03	<0.0001
	Tyloses–PCR	0.5	<0.0001
Bark (n = 32)	Discoloration–culture	0.1	0.003
	Discoloration–PCR	0.05	1
	Culture–PCR	0.004	0.002
	Discoloration–fungal structure	0.2	0.45
	Fungal structure–PAR	0.6	0.0005
	Fungal structure–PCR	1	0.3

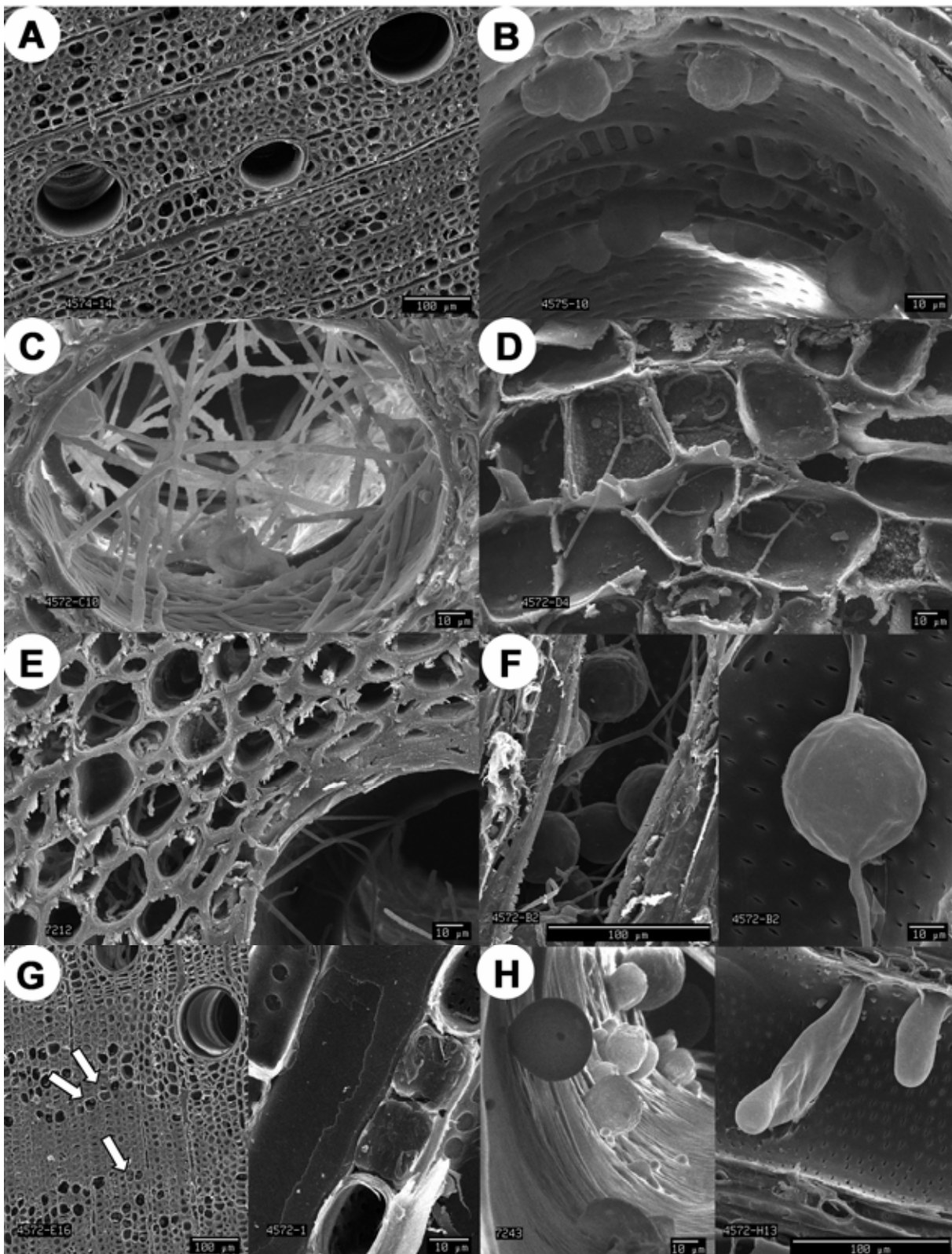
<sup>y</sup> Fungal structures include hyphae or chlamydo spores as observed with light microscopy or scanning electron microscopy.

<sup>z</sup> Fisher's exact test was used for the bark data because of the small sample size ( $\geq 25\%$  of the cells in the chi-square analysis had expected counts of less than 5).



**Log inoculation study: Effect of inoculum placement on infection spread.** *P. ramorum* readily infected sapwood even when inoculum was placed in the bark or at the cambium (Table 4 and Fig. 4). Eight weeks after inoculation, axial spread of infection was more extensive in the bark than in the sapwood when inoculated either in the bark or at the cambium. When sapwood

was inoculated, axial spread was as extensive in the sapwood as in the bark. Recovery from tissue by culturing and detection with PCR closely mirrored areas of discoloration. *P. ramorum* was recovered or detected from 90% of discolored sapwood samples ( $n = 163$ ), and from 89% of discolored bark samples ( $n = 84$ ) (data not shown). Interestingly, in these inoculated logs, *P. ramorum*



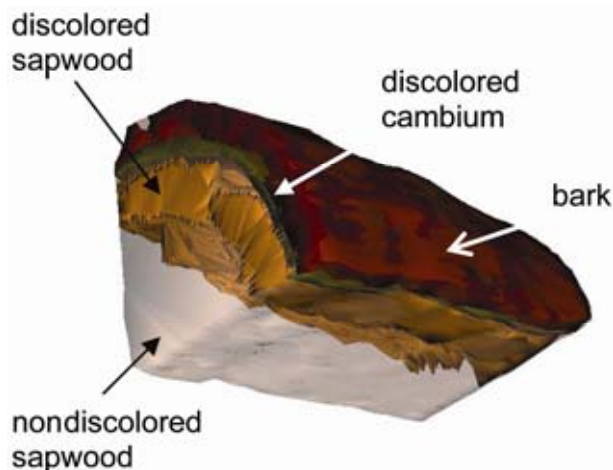
**Fig. 2.** Scanning electron micrographs of sapwood (A and B) not infected or (C through H) infected with *Phytophthora ramorum*. A, No hyphae in sapwood; B, tyloses in vessels; C, hyphae in vessels; D, hyphae in inner bark; E, hyphae in ray tracheid; F, chlamydospores in vessels; G, infected fiber cells (arrows); and H, tyloses in vessels.

was also recovered from 55% of asymptomatic sapwood tissue within 1 cm of discolored tissue, providing additional evidence that the pathogen is present beyond the discolored area. In the noninoculated controls, discoloration was restricted to the wound site itself, and was very dark in color unlike the grayish discoloration associated with *P. ramorum*-infected sapwood.

The depth of spread into the sapwood was very similar regardless of the site of inoculation. The mean depth of recovery or detection of *P. ramorum* in the sapwood did not differ significantly among inoculation treatments (3.0 cm from a bark inoculation, 3.3 cm from a cambium inoculation, and 3.3 cm beyond the point of sapwood inoculation). This represents a radial spread rate of 0.54 to 0.59 mm per day. *P. ramorum* was not detected in any of the noninoculated controls, and sapwood discoloration was darkly colored and clearly different than the grayish discoloration typical of *P. ramorum* symptoms.

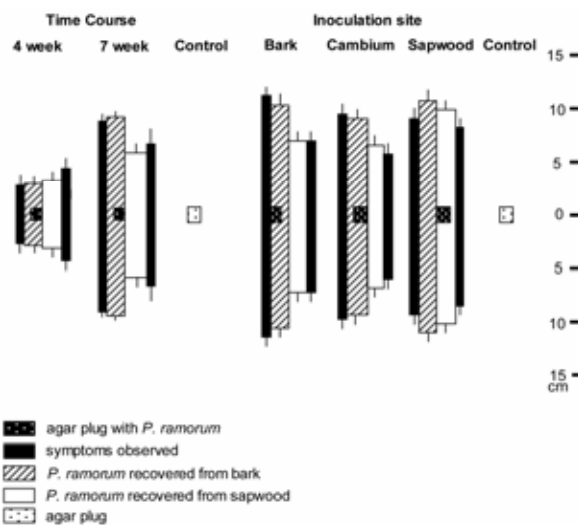
**Log inoculation study: Time course of infection spread.**

When logs were inoculated at the cambium, *P. ramorum* initially spread in the sapwood as rapidly as it did in the bark (Fig. 4). By week 7, the extent of infection was greater in the bark than the sapwood. Axial spread in this trial was similar to the analogous treatment (cambium inoculation) in the inoculum placement trial described above. The efficiency of recovery and detection was very similar to the previous trial.



**Fig. 3.** Model of one quarter of a bole section from infected tree 4572 showing areas of discolored bark and discolored sapwood indicative of infection by *Phytophthora ramorum*.

**Physiological studies.** Although sap flux sensors were installed on 7 July, rain and foggy weather in this coastal maritime climate prevented adequate transpiration to provide representative trends in  $J_s$  until the weather cleared on 11 July. The nearest weather station (Brookings, OR) recorded a maximum temperature 3.3°C greater on 11 July than the previous 4 days. Repeated measures analysis, taking into account the co-variance of sensors within trees, showed that sap flux did not consistently differ ( $P < 0.44$ ) between infected versus control trees during the course of the day. However, the ANOVA did show as expected, that sap flux differed significantly depending on the time of the day, i.e., treatment  $\times$  time showed a significant effect, ( $P < 0.0001$ ). Corresponding to our a priori hypothesis that peak sap flux would differ between treatment groups, a pair-wise comparison found peak sap flux values of infected trees to have been significantly reduced ( $P = 0.043$ ) by 42% compared to control trees (Table 5 and Fig. 5). In the laboratory we tested the conductivity ( $k_s$ ) of the wood from around the same sensors. The  $k_s$  of infected trees was also significantly reduced ( $P = 0.003$ ); it was only 37% of the value for wood from control trees (Table 5 and Fig. 6). The maximum



**Fig. 4.** Axial spread of *Phytophthora ramorum* above and below the inoculation site in logs. Bars indicate average spread (+/- standard deviation) for recovery/detection and symptom development in sapwood and bark in the time course experiment at 4 and 7 weeks (left) and in the inoculum placement experiment at 8 weeks after inoculation (right).

**TABLE 4.** Effect of inoculation site on symptom development and recovery or detection of *Phytophthora ramorum* 8 weeks later

Inoculation site	Axial spread (cm)						Radial spread (cm)	
	Symptoms <sup>u</sup>		P value <sup>w</sup>	Recovery/detection <sup>v</sup>		P value <sup>w</sup>	Symptoms	Recovery/detection
	Bark	Sapwood		Bark	Sapwood		Sapwood	Sapwood
Bark	21.8 ± 1.5 <sup>x</sup> n = 10	13.2 ± 1.6 n = 9	<0.0001 n = 9 pairs	20.1 ± 1.7 n = 7	13.5 ± 1.7 n = 7	0.004 n = 5 pairs	3.6 ± 0.5 n = 10	3.0 ± 0.5 n = 10
Cambium	18.6 ± 1.5 n = 10	11.5 ± 1.5 n = 10	0.0002 n = 10 pairs	17.6 ± 1.7 n = 7	12.4 ± 1.7 n = 7	0.014 n = 6 pairs	3.8 ± 0.5 n = 10	3.3 ± 0.5 n = 10
Sapwood	17.6 ± 1.6 n = 9	16.3 ± 1.5 n = 10	0.459 n = 9 pairs	20.6 ± 1.7 n = 7	18.8 ± 1.7 n = 7	0.357 n = 4 pairs	3.5 ± 0.5 n = 10	3.3 ± 0.5 n = 10
Sapwood (noninoculated control)	1.6 ± 0.2 <sup>y</sup> n = 10	6.1 ± 0.9 n = 10		0 n = 10	0 n = 10	0.6 ± 0.13 n = 10		0 n = 10
							P = 0.90 <sup>z</sup>	P = 0.89 <sup>z</sup>

<sup>u</sup> Symptoms in bark consisted of necrotic lesions; symptoms in sapwood consisted of grayish discoloration.

<sup>v</sup> Recovery/detection of *P. ramorum* refers to recovery by culturing on PAR selective medium or detection by diagnostic polymerase chain reaction (PCR).

<sup>w</sup> P values for axial spread are for analysis of variance using a hierarchical model of inoculation site, tissue type (bark or sapwood), the interaction between tissue type and inoculation site, and a random effect of location to account for paired bark and sapwood samples.

<sup>x</sup> Values are adjusted means (to account for missing data) ± standard error of the adjusted means.

<sup>y</sup> Symptoms from noninoculated controls consisted of dark streaking typical of a wound response. This treatment was excluded from the statistical analysis.

<sup>z</sup> For radial spread, P values are for ANOVA of symptoms and recovery/detection in sapwood as a function of inoculation site.

tension gradient did not differ significantly between control and infected trees (Table 5).

In the laboratory, removal of the outer bark confirmed that all of the sensors placed in infected trees were near cankers. In the infected trees, six of the nine sensors were placed in discolored sapwood, but three sensors had been placed in healthy-appearing tissue. *P. ramorum* was not recovered or detected from three of the sensor locations, but the pathogen was recovered from sapwood either below or above the sensor locations in all symptomatic trees. *P. ramorum* was not recovered or detected from any location in the control trees, nor were bark symptoms or discolored sapwood observed.

A difference in vessel characteristics between control versus infected trees could result in sap flow or  $K_s$  measurements that relate more to inherent differences in tree anatomy than to presence of the pathogen, however vessel diameter distributions, mean vessel diameters, and mean hydraulic vessel diameters were very similar for infected and control trees (Fig. 7 and Table 5). The proportion of vessel cross-sectional area as a proportion of total sapwood cross-sectional area also was not significantly different ( $P = 0.08$ ) between infected (3.47%) and control (4.18%) trees. Vessel cross-sectional area measurements may have been less owing to the abundance of tyloses. Unlike vessel diameters, which were measured individually, vessel cross-sectional area was measured by image analysis of axial sapwood sections. The program assigned areas of transmitted light as vessels and dark areas as nonconductive tissue. Vessel areas from this analysis may have been underestimated if partial occlusions by tyloses or fungal structures were assigned as nonconductive area. Because infected versus control trees were found to have 19.4 and 10.9% of vessels with tyloses, respectively, the actual cross-sectional vessel area for the two groups is likely to be more similar than was measured.

## DISCUSSION

This research shows that *P. ramorum* produces hyphae and chlamydospores in xylem tissue of tanoak and demonstrates that its widespread presence in sapwood is strongly associated with discoloration. In log inoculation studies, sapwood was infected readily, even in the initial stages of disease. Most importantly, results show that water transport in the bole is reduced when xylem is colonized by *P. ramorum*.

Discoloration is a good indicator of *P. ramorum* colonization of tanoak sapwood because the pathogen was recovered or detected from 83% of discolored tissue samples. However, discoloration likely underestimates the extent of infection because 40% of adjacent healthy-appearing tissues were also positive for *P. ramorum*,

and nearly all (18 of 19) of these microscopy samples also revealed hyphae and chlamydospores typical of *P. ramorum*. The pathogen is apparently present in tissue before discoloration occurs, suggesting that the discoloration is induced by infection.

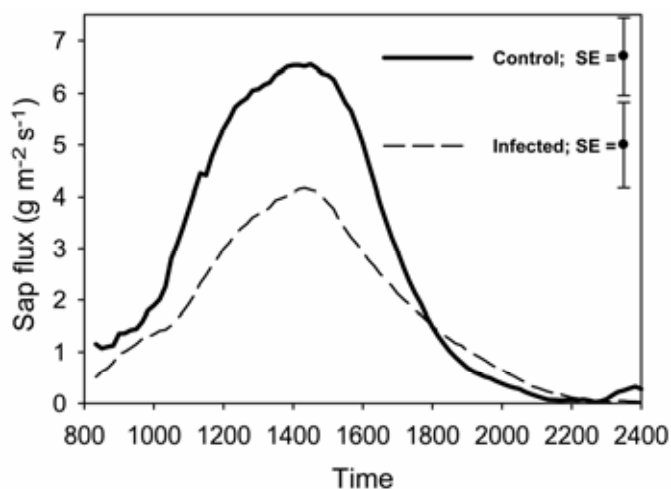


Fig. 5. Sap flux ( $J_s$ ) in naturally occurring *Phytophthora ramorum*-infected versus noninfected control trees in Curry County, Oregon during a 16-h period on 11 July 2005. Data are averaged from three infected trees and three noninfected trees. Bars indicate standard errors of the mean for each treatment.

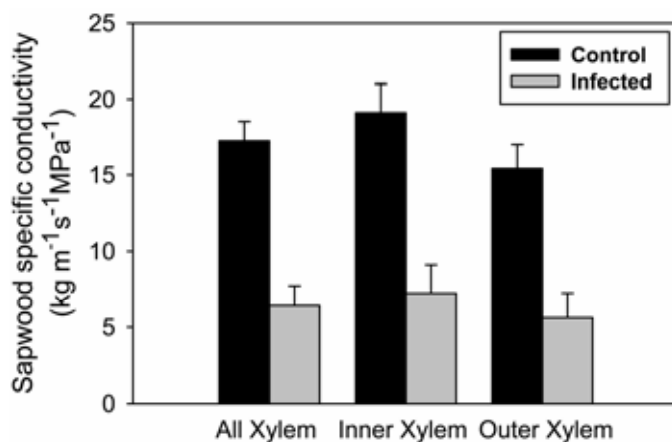


Fig. 6. Specific conductivity of sapwood  $k_s$  ( $\text{kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$ ), for all samples ( $n = 12$  in each group) or broken into inner xylem ( $n = 6$  in each group) and outer xylem ( $n = 6$  in each group). Bars indicate means  $\pm$  standard error.

TABLE 5. Summary of hydraulic and anatomical properties of trees infected by *Phytophthora ramorum* and noninfected control trees (values are means  $\pm$  standard error of the mean)

	Control	Infected	<i>P</i>
Peak $J_s^t$	6.55 $\pm$ 0.67 ( $n = 3$ )	3.83 $\pm$ 1.67 ( $n = 3$ )	0.047
$k_s^u$	17.3 $\pm$ 3.1 ( $n = 6$ )	6.4 $\pm$ 1.3 ( $n = 6$ )	0.003
Maximum tension gradient <sup>v</sup>	0.0274 $\pm$ 0.005 ( $n = 9$ )	0.0255 $\pm$ 0.008 ( $n = 8$ )	0.41
Mean vessel diameter, $\mu\text{m}^w$	102.9 $\pm$ 1.20 ( $n = 800$ )	102.9 $\pm$ 0.98 ( $n = 800$ )	0.98
Mean hydraulic vessel diameter, $\mu\text{m}^x$	129.1 $\pm$ 3.8 ( $n = 16$ )	124.8 $\pm$ 2.7 ( $n = 16$ )	0.30
Vessel cross-sectional area, % <sup>y</sup>	4.18 $\pm$ 0.09 ( $n = 27$ )	3.47 $\pm$ 0.12 ( $n = 27$ )	0.08
Tylosis occurrence, % <sup>z</sup>	10.9 $\pm$ 0.05 ( $n = 27$ )	19.4 $\pm$ 0.11 ( $n = 27$ )	0.04

<sup>t</sup> Peak sap flux ( $\text{g m}^{-2} \text{s}^{-1}$ ) is defined as the highest 50% of non-zero values (37 time periods).

<sup>u</sup> Specific conductivity ( $\text{kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$ ) based on two values per sensor indicative of inner and outer sapwood locations.

<sup>v</sup> Mean tension gradients ( $\text{MPa}^{-1} \text{m}^{-1}$ ) were calculated from the maximum sap flux at each sensor. ( $\text{MPa}^{-1} \text{m}^{-1}$ ) and means of the  $k_s$  values of the three samples taken near each sensor.

<sup>w</sup> Mean vessel diameter is based on average width measurements for 50 vessels from one outer sapwood and one inner sapwood samples for 16 of the 18 sensor locations.

<sup>x</sup> The diameter of a vessel that would supply the observed conductivity is calculated from the vessel diameter distribution as the as  $\Sigma d^5 / d^4$  from one outer xylem and one inner sapwood sample for 16 of the 18 sensor locations.

<sup>y</sup> Average vessel cross-sectional area as a proportion of total cross-sectional area of sapwood.

<sup>z</sup> Average frequency of tylosis occurrence was determined for 100 vessels for each sapwood sample.



The discoloration itself could come from any of several sources: the opaque material observed in the ray cells (which resemble gels and gums produced by hosts in response to fungal pathogens), and/or materials released from host cells damaged by *P. ramorum*. *P. ramorum* produces two elicitors that result in the disruption of cell walls and cell membranes of tanoak and other hosts (D. Manter, *personal communication*).

Tomography of infected trees allowed visualization of the pattern of discoloration within the tree. It revealed the vertical alignment of the discolored areas and showed that discolored (infected) sapwood was often separated from infected bark by a layer of nondiscolored sapwood. While a valuable tool, tomography was extremely labor-intensive. The technique would be more useful if it could be based on a specific colorimetric assay or fluorimetric probe for *P. ramorum* rather than discoloration and confirmation by culturing and PCR detection of the pathogen.

The presence of *P. ramorum* in xylem introduces additional possibilities for symptomatology, pathogenesis, and disease spread. Obstructions in the water transport system that directly or indirectly decrease water transport could lead to rapid death of branches (flagging) or crowns during periods of water deficits, contributing to the appearance of sudden tree death and decreasing plant vigor through decreased photosynthetic gain. The presence of *Phytophthora* in xylem could also enhance the rapid transport and delivery of elicitors to actively growing tissues throughout the tree. In addition, growth of *P. ramorum* through vessels (all of which are nonliving) might provide the pathogen with a “superhighway” for rapid vertical spread within the tree, providing access to food sources from nearby living parenchyma cells while affording some protection from full-scale host responses. Xylem infection might also provide a link between bark cankers as suggested by Brown and Brasier (2) or, in theory, provide a link between twig infections in the canopy and bole infections, although evidence for these phenomena in tanoak is lacking. The infected trees in this study were mostly in the intermediate stages of disease (green canopy but with bleeding cankers). It was not possible to determine in specific tissues which occurred first, bark colonization or sapwood colonization. The time course study in inoculated logs indicated that *P. ramorum* spread as fast in sapwood as it did in bark during the first 4 weeks, but by 7 weeks, it had spread further in bark. A time course study of infection in living trees using localized inoculum would help clarify the sequence of events and determine the epidemiological ramifications. Sporangia applied to nonwounded tanoak bark surfaces can initiate infection (J. Davidson, and

D. Rizzo, *personal communication*), and dissection of recently naturally infected trees suggests penetration of bark tissues from the outside in (E. Hansen and J. Parke, *unpublished data*). Perhaps canker enlargement is speeded, however, by axial growth of hyphae in vessels.

The water transport studies together with the anatomical observations, suggest that water transport in the infected trees was decreased by obstructions caused by fungal hyphae, chlamydospores, tyloses, and probably embolisms. The specific conductivity tests on excised wood segments show how much water will cross a transverse area of wood per unit time with a given driving force. In the specific conductivity tests, the embolisms which were present *in vivo* have been removed. The lower specific conductivity of the sapwood from infected trees, given the lack of significant differences in wood anatomy as compared to sapwood from control trees, is strongly suggestive that fungal material (hyphae and/or chlamydospores) and/or tyloses were blocking some vessels in the wood of the infected trees.

The lower sap flow in infected trees showed at the whole-tree level, *in vivo*, that less water was ascending per cross-sectional area of wood in diseased trees. Sap flow measurements integrate all the factors in the field and do not require knowledge of factors such as the driving force for water movement, soil moisture, or transpiration rate. The lower sap flow in infected trees could be caused by any combination of the following factors: decreased specific conductivity (observed) because of physical blockages such as tyloses (observed) and fungal material (observed), physical blockages by embolisms in the vessels (implied but not assayed), lower driving force for water movement due to lower total transpiration in infected than diseased trees (not likely to have occurred, since crown size was larger in infected than control trees), or lower water availability to the plant (not likely to have occurred, since *P. ramorum* is not known to cause root infection on mature trees). The increased embolism in infected trees was not measured, but is implied by the higher incidence of tyloses in infected than control trees. Tyloses appear to form only after a vessel has undergone embolism (4), and can form within days if an injury occurs during the growing season (16).

It is not surprising that infected and control trees showed no significant differences in their xylem tension: the tension gradient in a woody plant is often under physiological control through stomatal control of transpiration or through shedding of leaves (3). This result suggests strongly that the increased incidence of tyloses in infected trees was caused by fungal perforations of the vessel walls that allowed air-seeding and embolism of vessels, and not by higher xylem tensions in infected trees.

One could ask whether tylosis formation is an inducible defense to impede the spread of fungus through the stem. This phenomenon seems unlikely, as it would require that the tree have a mechanism to increase air-seeding through increasing the tension gradient in the stem (not observed) or making new holes or enlarging existing holes (such as pores in pit membranes). The only living cells within the xylem are axial and ray parenchyma cells. If tyloses were formed as an inducible defense, these parenchyma cells would have to exude cellulytic enzymes. Thus *P. ramorum* would affect the parenchyma cells, which would then cause holes in the vessel walls, causing embolisms and then tyloses. It is much more likely that *P. ramorum* itself produces the holes in the vessel walls that lead to embolism and reduced xylem conductivity.

The midday peaks of sap flux as well as total sap flux (the area under the curves in Figure 5) reported here should represent near growing season maximums for both infected and control trees given the long summer day length and the ample soil-water supply on July 11. The maximum sap flux densities we recorded ( $\approx 6.5 \text{ g m}^{-2} \text{ s}^{-1}$ ), are substantially lower than the maximum sap fluxes found by Hirose et al. (12) for another *Lithocarpus* sp. ( $\approx 45 \text{ g m}^{-2} \text{ s}^{-1}$ , measured directly on an excised tree in water, with

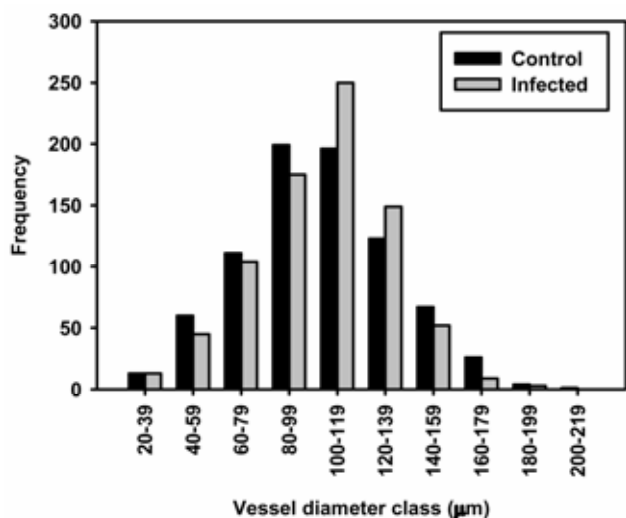


Fig. 7. Frequency distribution of vessel diameters of control and infected by *Phytophthora ramorum* trees ( $n = 800$  per group).

water taken up by the cut end). Much of this difference surely owes to the lack of resistances within the roots and across the root-soil interface in the experiment by Hirose et al. (12) because a comparison of anatomical measurements shows very similar mean vessel diameters and percent conductive xylem area. All else equal, sap fluxes are lower in trees with proportionally more sapwood. This may be true since there was no discernible heartwood in any of the six trees sampled.

Canker-causing *Phytophthora* species are generally believed to cause disease by killing phloem and impeding transport of photosynthate. There have been few examples of *Phytophthora* species that colonize secondary xylem (5,7,17) and in some of these cases xylem colonization apparently did not occur until after trees were killed by the pathogen (23). However, recent findings in the United Kingdom indicate that xylem colonization of living tree hosts by canker-causing *Phytophthora* species may be more widespread than recognized previously (2). Our research provides evidence that xylem colonization by *Phytophthora ramorum* reduces water transport in the bole and suggests a physiological basis for sudden oak death in tanoak. Vascular wilt pathogens “invade the xylem of roots and stems and produce diseases primarily by interfering with the upward movement of water through the xylem” (1). *P. ramorum* can be a vascular wilt pathogen on tanoak and perhaps other bole canker hosts, forcing us to reconsider the distinction between canker diseases and vascular wilts.

#### ACKNOWLEDGMENTS

This work was made possible by grant 2005-JV-052 from the United States Department of Agriculture – Forest Service, Pacific Southwest Research Station and a special USDA grant to OSU for wood utilization research. We thank A. Kanaskie and M. McWilliams, Oregon Department of Forestry, and South Coast Lumber for their cooperation in this study. N. Ochiai was instrumental in applying the 3D imaging software. S. Wang, J. Engle, and S. Dunham provided excellent assistance for many aspects of the project. We thank B. Ferguson for valuable ideas and discussions concerning tyloses.

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