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Histology of *Phytophthora ramorum* in *Notholithocarpus densiflorus* bark tissues†

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Abstract

Colonisation of *Notholithocarpus densiflorus* (Hook. and Arn.) Rehder tissues by *Phytophthora ramorum* Werres, De Cock & Man in't Veld is not well understood. The pathogen is able to colonise nearly all tissues of this host but it is unclear how a tree is ultimately killed. In this research, *P. ramorum* infected *N. densiflorus* bark tissues were examined using various microscopic techniques to better understand the role of bark infection in killing a tree. Host responses to infection were detected by histological methods in conjunction with examining *P. ramorum* colonisation. Results of this work indicate that the pathogen can colonise nearly all *N. densiflorus* bark tissues but that phellogen and parenchyma of the inner bark are the most frequently and densely colonised. Pathogen specific elicitin labelling of *P. ramorum*-infected *N. densiflorus* sprouts caused hyphal cell walls to fluoresce in plant tissues, allowing specific identification of hyphae. Findings of this research show that nearly all bark tissues are capable of being colonised, that this host responds to infection with callose deposition, tissue discoloration, and cell collapse; and that elicitins are present in cell walls of hyphae in infected bark tissues.

Keywords: histopathology; immunofluorescence; pathogenesis; phloem; sudden oak death; tanoak.

† *Based on a paper presented at the fifth meeting of the IUFRO working party S07-02-09, Phytophthora Diseases in Forests and Natural Ecosystems, 7 – 12 March 2010, Auckland and Rotorua, New Zealand.*

Introduction

Phytophthora ramorum Werres, De Cock & Man in't Veld (2001), the organism responsible for sudden oak death (Rizzo et al., 2002), continues to cause extensive mortality to tanoak, *Notholithocarpus densiflorus* (Hook. and Arn.) Rehder (Manos et al., 2008) along the coast of the western United States of America (USA). The results presented here are part of research investigating pathogen colonisation and pathogenesis in tanoak bark.

The most apparent symptom of infection on *Notholithocarpus densiflorus* is localised "bleeding" on the bole of the tree. Dark-coloured liquid exudes from small cracks in the bark, indicating more extensive necrotic lesions in the underlying bark tissues. The extent of necrosis is only visible after the outer bark has been scraped away. It has been assumed that trees are killed by extensive death of bark and cambium tissues and disruption of phloem transport (Parke et al., 2007). However, recent work has shown that *Phytophthora ramorum* also colonises the sapwood of *N. densiflorus* trees and reduces hydraulic conductivity of the xylem

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(Parke et al, 2007; Collins et al., 2009). The relative importance of xylem and phloem colonisation in pathogenesis has yet to be properly evaluated.

In many tree-canker diseases caused by *Phytophthora* species, the pathogen preferentially colonises the cambium and neighbouring inner phloem and outer xylem tissues (Tippett et al., 1983; Robin, 1992; Davison et al., 1994). During later stages of infection, *Phytophthora* may move further into the phloem or xylem, depending on the type of disease caused (Davison et al., 1994; Robin, 1992; Brown & Brasier, 2007; Davison, 2011). In the stems of *Rhododendron* spp., *P. ramorum* colonised all tissues except the cambium, primary phloem and epidermis. Overall, hyphae were most frequent in xylem tracheids. Hyphae grew intercellularly and intracellularly in the pith and cortical parenchyma, but mostly intracellularly in other tissues (Pogoda & Werres, 2004).

Parke et al., (2007) showed that *P. ramorum* infected sapwood of *N. densiflorus*. Hyphae were abundant in xylem ray parenchyma and it was hypothesised that they gained access to sapwood tissues by travelling through rays from bark tissue. Hyphae were also found in vessels and fibre tracheids of the sapwood, penetrating 3 cm deep on average. Abundant tyloses were observed in the vessels of infected tissues and sapwood conductance was disrupted. *Phytophthora ramorum* was also found to infect the sapwood of *Quercus* and *Fagus* species (Brown & Brasier, 2007).

Similar histopathological studies of *Phytophthora lateralis (*Tucker and Milbrath*),* the closest known relative of *Phytopthora ramorum*, in roots of *Chamaecyparis lawsoniana* ((Murr.) Parl.) showed that hyphae grew inter- and intracellularly in the phloem, being most concentrated in sieve cells and parenchyma cells of the functional phloem in the most susceptible plants and were seen only incidentally in xylem (Oh & Hansen, 2007).

Plants respond to infection by pathogens, including species of *Phytophthora*, in a variety of ways. These may include collapse and necrosis of cells, and the formation of barriers to further pathogen colonisation, including localised deposition of callose. *Phytophthora ramorum* in *Rhododendron* spp. caused necrosis of cortical parenchyma, cambium and collenchyma tissues marked by severe discoloration (Pogoda and Werres 2004). Callose, a polysaccharide made up of glucose molecules connected by β-1,3 linkages, is formed in various cells and tissues of healthy, wounded, and pathogen-infected plants (Aist, 1976; Mullick, 1977). In a healthy plant, callose usually occludes the sieve pores of sieve elements during the autumn (fall) and winter months while the plant is dormant, as well as sieve pores of older, non-conducting sieve elements (Fink, 1999). The occlusion of sieve pores with callose also occurs with pathogen introduction, and can occur within minutes, being among the most rapid of plant defence responses. It is more difficult for the pathogen to penetrate regions where callose is deposited so callose deposition helps the host to isolate the pathogen (Fink, 1999).

Elicitins are a class of small molecular-weight proteins produced only by *Phytophthora* and *Pythium* species. Their major biological role is presumed to be to transport sterols (Kamoun et al., 1993; Tyler et al., 2002). However, elicitins were first discovered due to, and have been best studied for, their ability to act as elicitors of host defence in incompatible disease interactions (Kamoun et al., 1993; Keller et al., 1996; Sasabe et al., 2000; Tyler et al., 2002). Tools have now been developed to label elicitins *in situ*. A fluorescently labelled antibody specific for *Phytophthora ramorum* elicitins was developed by Dan Manter, United States Department of Agriculture (USDA) Agricultural Research Service (ARS) at Fort Collins, Colorado in the USA. The antibody was used in the studies described here to positively identify and visualise *P. ramorum* hyphae in infected *N. lithocarpus* tissues.

In conducting histological studies on bark tissues, it is important to understand the basic anatomy of bark (Figure 1). In trees, “bark” includes all tissues outside of the vascular cambium. The “inner bark” with the youngest tissues and still translocating vessel elements lies from the vascular cambium out to the first layer of periderm. The “outer bark” consists of all tissues of the youngest periderm and other periderm layers formed previously. As a tree ages, it may produce multiple periderms layered alternately with secondary phloem tissue layers. Each periderm is composed of three tissue types, the phellogen or cork cambium, the phelloderm and the phellem or cork. The phellogen is a meristematic tissue from which the other two tissues arise. The overall appearance of the phellogen is similar to the vascular cambium. Phellem, the tissue produced to the outside of the phellogen, is comprised of several layers of heavily suberised and lignified dead cells, appearing oblong and radially shortened in transverse section. This layer serves as protection from extreme weather conditions, pests, and pathogens. The phelloderm, the tissue produced to the inside of the phellogen, is comprised of radially arranged parenchyma cells, polygonal to rounded in shape with intercellular spaces that vary in size and abundance among species. Cell walls may or may not be thickened (Esau, 1969). The outermost layer(s) of phelloderm may contain photosynthetic chloroplasts in some thin-barked species (Alekseev et al., 2007), including tanoak.

The “inner bark” is composed of secondary phloem, only the innermost portion of which contains conducting phloem. Cell types of the inner bark include: sieve tube elements and their associated companion cells (in Angiosperms); ray parenchyma; phloem parenchyma;

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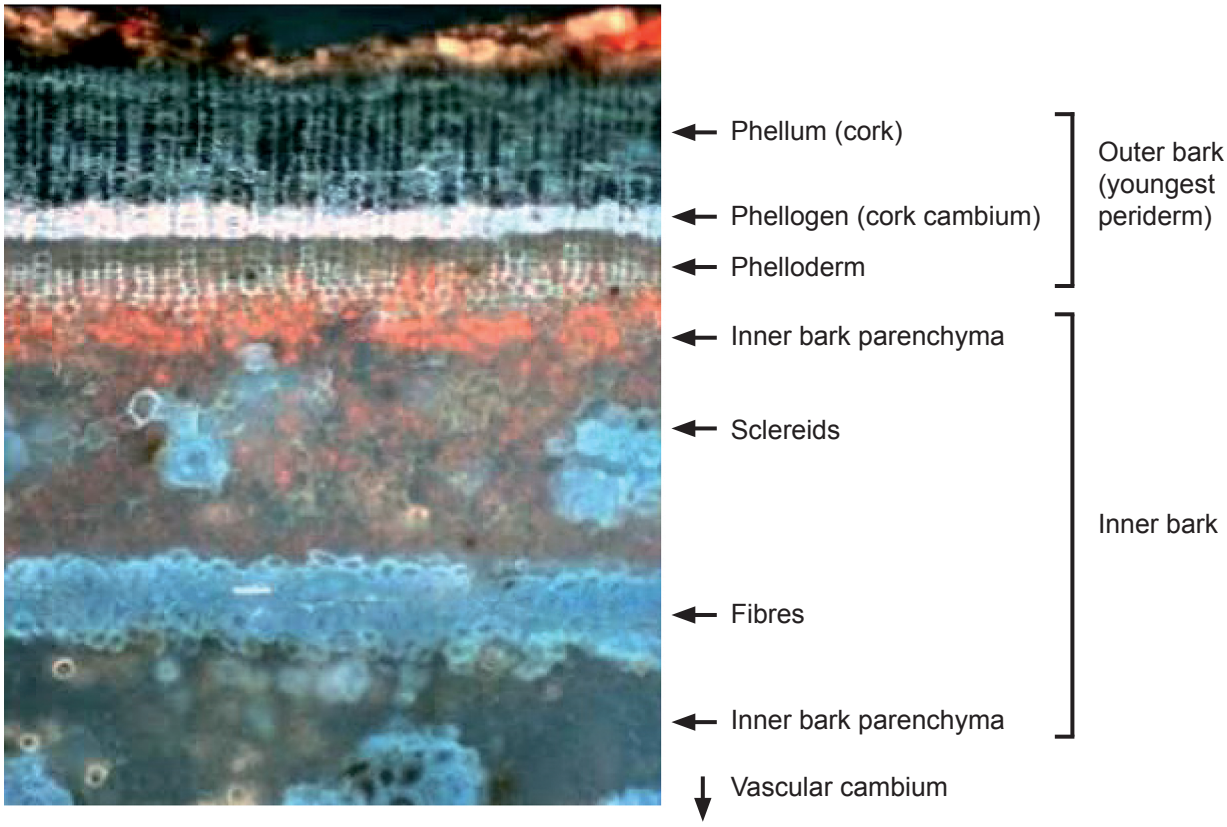

FIGURE 1: Autofluorescence image of an uninfected *Notholithocarpus densiflorus* bark cross section from the inner bark to the youngest periderm. Cambium is not shown.

sclereids; and fibres. A sieve tube element is a conducting cell. It is tubular and elongated and possesses sieve areas on its elongated walls as well as its end walls. While sieve tube elements contain some organelles, the majority of cellular functions for the sieve tube element take place in the adjacent, and much smaller, companion cell, which is connected to the sieve tube element by plasmodesmata. These two cell types occupy the large majority of area within the functional phloem, with some interspersed fibres and other parenchyma.

The two types of parenchyma cells found in inner bark aside from sieve elements and companion cells are the phloem parenchyma and the ray parenchyma. Phloem parenchyma can store a variety of compounds including sugars, lipids, and defence compounds, and can transport them locally. Ray parenchyma mainly serve to compensate for xylem girth increases but can also transport materials locally.

Sclereids and fibres, collectively known as sclerenchyma tissues, are the only two types of cells with secondary walls in *N. densiflorus* bark. These cells usually have heavily lignified secondary walls and are dead at maturity. Sclereids in *N. densiflorus* are polygonal to rounded, whereas fibres are elongated. Sclereids can also be distinguished from fibres by greater number of pits in their cell walls. Both cell types usually occur in clusters but can also occur singly. Sclerenchyma tissues provide structural support to bark tissue.

This paper details a study of colonisation of *N. densiflorus* bark tissues by *P. ramorum,* which has not been previously examined histologically. We investigated the presence of *P. ramorum* in tanoak bark primarily using fluorescence microscopy and by immunolabelling of *P. ramorum* elicitins using a fluorescently labelled antibody to highlight hyphae. Confocal microscopy and scanning electron microscopy were also used in order to better track growth of hyphae between cells, as well as callose deposition and wound periderm formation (Botts, 2009).

Methods

Phytophthora ramorum cultures

Two *P. ramorum* isolates, Oregon State University (OSU) 2018.1 and OSU 2027.1, were used. These were originally isolated from naturally infected *N. densiflorus* trees in Curry County, Oregon, USA. Pure cultures of *P. ramorum* were grown and maintained on BBL corn meal agar (Becton Dickinson & Co., Sparks MD, 21152).

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Tanoak sample collection

Three types of tanoak sample were collected, as follows:

1. Infected bark tissue came from naturally infected tanoak trees. In January, 2007, five symptomatic infected tanoak trees, 10 – 46 cm diameter, were sampled at each of three infested sites in Curry County, Oregon. Infected trees had green crowns or were recently dead and all still had well-defined bark lesions marked by bleeding spots. The presence of *P. ramorum* was confirmed by isolation on selective agar medium followed by microscopic examination of colonies for characteristic features (Reeser et al., 2008). Healthy bark tissue for comparison came from two healthy trees at each of the sampling sites.

2. Healthy tanoak sprouts 20 – 30 cm long and <1 cm diameter were collected in January 2009 from Curry County, Oregon, for artificial inoculation. Sprouts came either from the base of trees or the tips of branches.

3. Tanoak logs (1 m long and 10 – 20 cm diameter) used for artificial inoculation were cut in January 2007 from healthy tanoak trees from outside of the *P. ramorum* quarantine area in Curry County Oregon. These were inoculated as described below.

Artificial inoculation

Twelve tanoak log sections were inoculated. A cork borer was used to create three holes to the cambium in each log, which were filled with an agar plug. Each log was inoculated with agar plugs bearing either isolate OSU 2018.1, OSU 2027.1, or a sterile agar plug as a control. Holes with agar plugs were covered with damp cheesecloth then foil attached with tape. Log ends were sealed with tree wax and logs were stored before and after inoculation in plastic bags at room temperature. Logs were inoculated in mid February 2007, and harvested seven weeks later. Active growth of *P. ramorum* was confirmed by isolation on selective agar medium followed by microscopic examination for identifying characteristics of this species as well as by polymerase chain reaction (Winton & Hansen, 2001).

Healthy sprouts were inoculated by placing cut ends in a suspension of *P. ramorum* zoospores. Zoospore suspensions were made up of an equal mixture of *P. ramorum* isolates OSU 2018.1 and OSU 2027.1. Mixed isolates were used to compensate for slight differences in maturity of individual isolates on inoculation days. Separate *P. ramorum* cultures were grown on V8 agar for 2-3 weeks until many sporangia had formed on the surface of each of 8 – 10 plates. Approximately 5 mL deionised water was poured on top of each plate and swirled to detach sporangia. The water was poured off and collected into one beaker. Sporangial suspensions were incubated at 4 °C for 1 – 1.5 hrs, then at room temperature for 45 minutes to trigger zoospore release. Zoospore concentrations in the combined suspension were adjusted to a total of 10^4/mL. Sprouts were inoculated by immersing cut ends in zoospore suspension to a depth of 1.5 – 2 cm. Sprouts were left in zoospore suspensions for two weeks before being removed for sampling. The lesion on each sprout usually extended several cm up the stem from the water line of the zoospore suspension. Sprout inoculations were performed twice, with a total of 33 replications and 8 control inoculations placed in sterile water.

Analytical samples

Areas to be sampled from naturally infected trees and or artificially infected logs were identified by removing outer bark to reveal canker margins. Samples were removed directly adjacent to an exposed lesion margin using a chisel to cut a rectangular section of intact bark, going all the way to the cambium. Each sample contained the lesion margin along with healthy and damaged tissue on either side of it. Healthy control samples were collected within 5 cm of the control inoculation site. For sprouts, a 1.5 cm section of stem with the lesion margin in the centre was cut out with a razor blade; controls were sampled at the water line.

Fixation

All sprout samples were placed immediately in Formalin-Acetic-Alcohol (FAA) solution (50% v/v ethanol (EtOH, 95%), 5% v/v glacial acetic acid, 10% v/v formalin (37% formaldehyde), and 35% v/v distilled H_2O). Samples to be immunostained for elicitins were immediately immersed and stored in FAA. To dehydrate samples in FAA, they were washed in deionised water two times for 30 minutes each then transferred through an ethanol concentration series to 100% EtOH.

Samples to be used for light- and fluorescence microscopy were stored in a 50 : 50 v/v EtOH/ glycerol solution to both kill and fix cells while also preserving autofluorescence.

Embedding with polyethylene glycol

All samples (except sprout samples to be elicitin labelled) were removed from their storage solution and were passed directly through a series of four decreasing dilutions of 1500 molecular weight polyethylene glycol (PEG) at 50 °C. Samples were held in each dilution for one day. Samples were then immersed in 100% PEG twice, using new PEG the second time.

Sprout samples to be elicitin labelled were removed

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from the FAA storage solution and passed through three increasing concentrations of 1000 PEG in EtOH, starting at equal parts PEG and ETOH and ending in 100% 1000 PEG. The last step was repeated with fresh PEG. Embedding was carried out at 40 °C to prevent protein degradation. Sprout samples were held in each dilution for 2 hours.

Sectioning

Samples stored in FAA solution or 50 : 50 EtOH/glycerol solution that were not embedded in PEG were washed for at least 2 h in either phosphate buffered saline (PBS) or water prior to sectioning on the microtome. Samples embedded in PEG were not washed prior to sectioning. Samples were sectioned using a sliding microtome to between 10 and 60 µm thickness. Some samples were hand sectioned to an estimated 40 to 80 µm.

Staining

Fixed sections (Mueller et al., 2004) were used for calcofluor staining. The calcofluor reagent was prepared by dissolving calcofluor white (0.05% w/v) in 0.5 M Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) buffer at pH 8. Sections were washed thoroughly in distilled H_2O then transferred to a clean, dry petri dish and covered with 1-2 drops of calcofluor reagent for 5 minutes, flooded with distilled H_2O and swirled for several seconds to rinse. Sections were washed three more times before mounting in distilled H_2O on slides. Six naturally infected field samples, seven artificially infected log samples, and three artificially infected sprout cutting samples with matching uninfected control samples were stained with calcofluor and viewed with fluorescence microscopy.

Aniline Blue stain (0.005% w/v aniline blue in 0.15 M K_2HPO_4 at pH 8.2) was prepared according to Ruzin (2001). Sections were stained on slides for 10 – 15 min. Aniline blue stains cellulose, callose, and other β-1,3 glucans (YuanTih & YuLing, 2004)

Immunofluorescent labelling of elicitins

Methods for immunofluorescent labelling of elicitins were modelled after those used by Brummer et al., (2002). Thirty-eight *P. ramorum* infected samples were treated by the immunofluorescent elicitin labelling procedure including: 27 inoculated sprout samples; nine inoculated log samples; and two infected field samples. Four uninfected sprout samples, and two field controls were also treated by this method. Samples stored in FAA were washed overnight in PBS, pH 7.4 and sectioned with a microtome. To prevent non-specific labelling, sections were first treated with a blocking solution containing 3% bovine serum albumin (BSA) in PBST (PBS with 0.2% Tween-20) for 30 minutes at room temperature. Next, they were incubated in a 1:500 solution of anti- elicitin rabbit serum in PBS and 1% BSA for 2 h at 37 °C. The sections were then washed well in PBST four times for 1 – 2 min each time followed by incubation in a 1 : 200 diluted solution of CY2 labelled goat anti-rabbit IgG in in PBS and 1% BSA for 1 h at 37 °C. They were again washed well four times with PBST.

Microscopy

The primary microscope used for both fluorescence and light microscopy was a Zeiss Axioskop (Oberkochen, Germany). For calcofluor-stained material a filter cube with excitation at 390 – 420 nm and emission at 450 nm was used. Plant cell walls and *Phytophthora* cell walls both appeared a bright whitish blue. For aniline blue-stained material, a UV filter with excitation at 345 nm and emission at 425 nm was used. Immunofluorescent-labelled sections were observed under the microscope using a filter cube with excitation at 450 – 490 nm and emission at 515 nm.

An FEI Quanta 600F Scanning Electron Microscope (Hillsboro Oregon) was used to view hyphae and other structures in bark tissues. Samples for Scanning Electron Microscopy (SEM) were fixed in 50 : 50 EtOH and glycerol and embedded in 1500 PEG, and included one artificially infected log sample, two naturally infected field samples, one artificially infected sprout, and one field control bark sample.

Results

Recognising *Phytophthora ramorum* in tanoak bark

Tree bark, especially dead outer bark and the necrotic tissues of diseased or wounded inner bark, is often colonised by a variety of fungi. Distinguishing target microorganisms (*P. ramorum* in this case) from saprophytic colonisers is a challenge. In our work, we relied on several tests to identify hyphae in the bark, we:

1. isolated (or reisolated) *P. ramorum* from all samples before histological examination;
2. contrasted hyphae observed in symptomatic bark with hyphae in bark from control trees;
3. compared morphology of hyphae in bark with hyphae in pure cultures of *P. ramorum*; and
4. successfully stained some samples with a *P. ramorum*-specific elicitin immunostain.

Hyphae that were considered to be *P. ramorum* were coenocytic and typically 2 – 7 µm in diameter, but reached up to 10 µm. Fluorescence of hyphae with these characteristics in samples successfully treated with the specific elicitin immunostain confirmed their identity as *P. ramorum* (Figure 2). Unfortunately the

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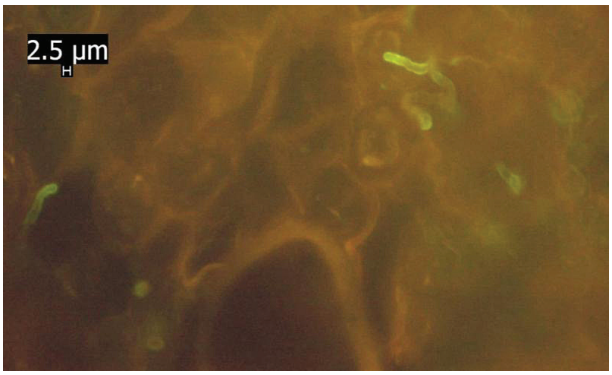

FIGURE 2: Elicitin production in hyphal cell wall. Fluorescence micrograph of a cross section of an elictin immunostained bark sample from a *Phytophthora ramorum* artificially infected sprout. Excitation filter 450 – 490 nm and emission at 515 nm.

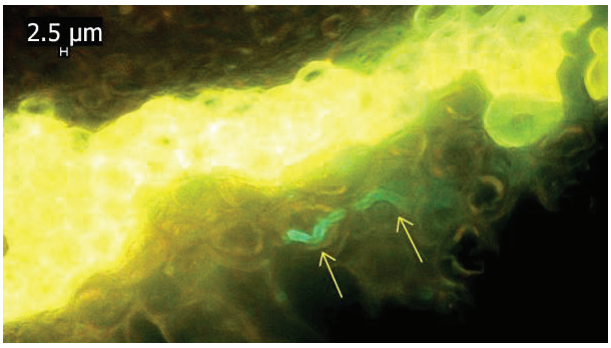

FIGURE 4: Elicitin immunostained hyphae in the parenchyma and phelloderm of inner bark. Fluorescence micrographs of cross sections of elictin immunostained bark samples from *P. ramorum* artificially infected sprouts. Excitation filter 450 – 490 nm and emission at 515 nm. Hyphae fluoresce green with elicitin immunostaining. Hyphae growing cell to cell through cell walls are marked with arrows.

elicitin immunostain method resulted in high levels of background fluorescence in most samples and hyphae were not visible. Hyphae that were generally 1.5 µm or less and/or had frequent crosswalls were not considered to be *P. ramorum*.

Colonisation of inner and outer bark tissues

Phytophthora ramorum hyphae were seen in all outer and inner bark tissues of infected samples, except fibre and sclereid tissues. Hyphae in the parenchyma and phelloderm of the inner bark are illustrated (Figure 3). Colonisation was primarily intracellular (Figure 4). Scanning electron microscopy allowed for visualisation of hyphae passing from cell to cell.

Hyphae appeared to penetrate host cell walls directly in some cases (not shown) but, they also appeared to grow through the pits connecting adjacent cells, as seen by SEM in Figure 5.

Hyphae were seen most frequently in the phelloderm and parenchyma cells in the outer part of the inner bark. Hyphae in these tissues grew vertically, horizontally, and radially, as seen with calcofuor fluorescence in Figure 6. Hyphae grew primarily intracellularly in these tissues (Figure 7) but, intercellular hyphae were also observed. The second most common place that hyphae were seen was in parenchyma tissues just inside of the first layer of sclereids and fibres encountered going from the outer to inner bark, beyond the discoloured, unhealthy looking region.

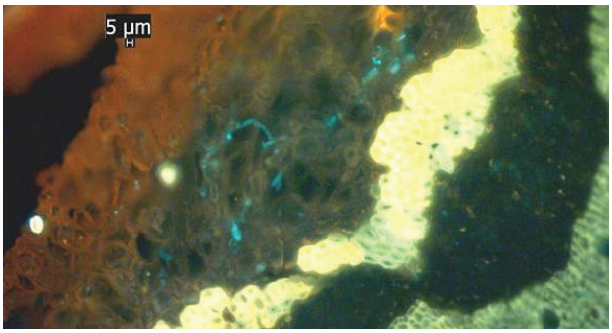

FIGURE 3: Elicitin immunostained hyphae in the parenchyma and phelloderm of inner bark. Fluorescence micrographs of cross sections of elictin immunostained bark samples from *Phytophthora ramorum* artificially infected sprouts. Excitation filter 450 – 490 nm and emission at 515 nm. Hyphae fluoresce green with elicitin immunostaining but appear blue due to photo processing.

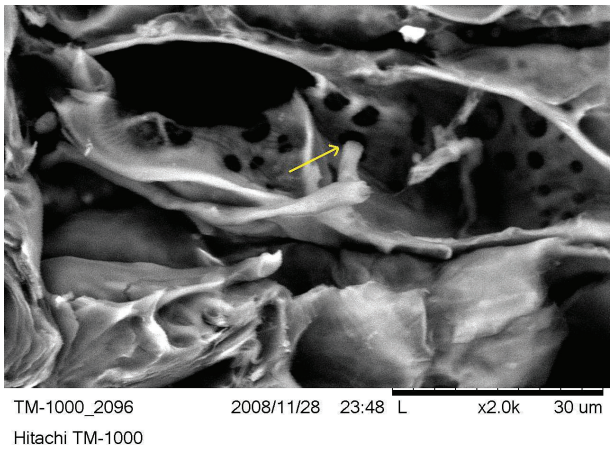

FIGURE 5: Hyphae growing through plasmodesmata. Scanning electron micrograph of a cross section of a *Phytophthora ramorum* infected bark sample collected from a naturally infected *Notholithocarpus. densiflorus* tree.

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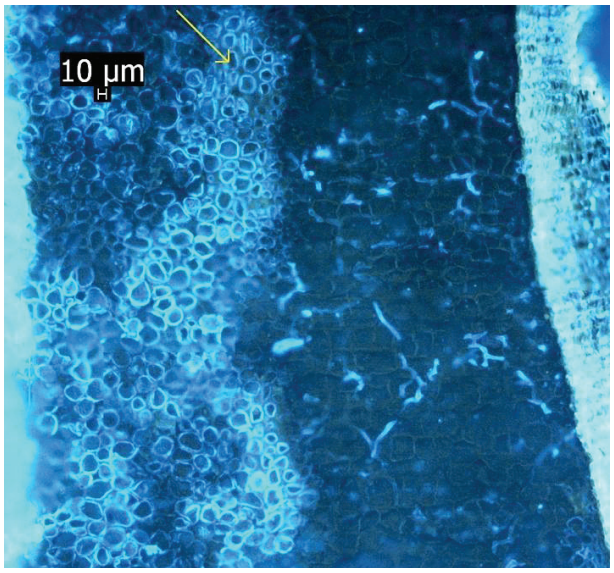

FIGURE 6: Longitudinal section of *Phytophthora ramorum* infected bark with hyphae growing radially and vertically: Fluorescence micrograph of a calcofluor stained bark sample from a *Notholithocarpus densiflorus* log artificially infected with *P. ramorum*. Hyphae are in phelloderm and parenchyma of the outer part of inner bark.

Again, hyphae here were primarily intracellular and grew in every direction (not shown). Staining with aniline blue was used to visualise colonisation of tissues of the innermost bark, in the region of conducting phloem. Only a few hyphae were observed. Hyphae were seen inside a stained sieve tube element (Figure 8). Hyphae were also seen in ray parenchyma cells, and in some samples cambium and

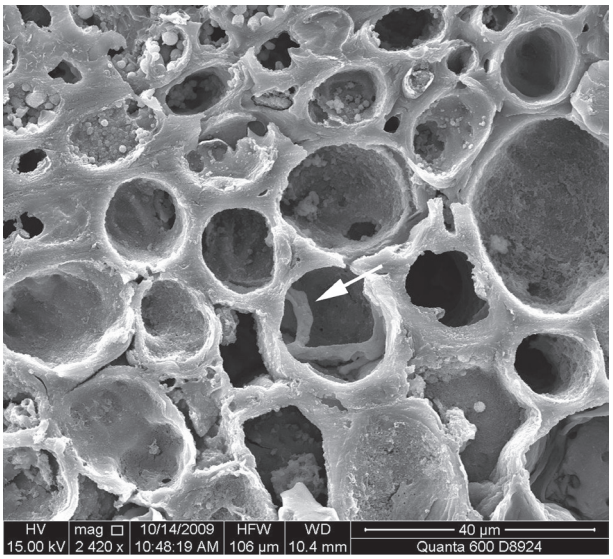

FIGURE 7: Hyphae (arrow) in parenchyma in a *Phytophthora ramorum*-infected sprout cutting. Scanning electron micrograph of a cross section of a bark sample from a *P. ramorum*-infected sprout.

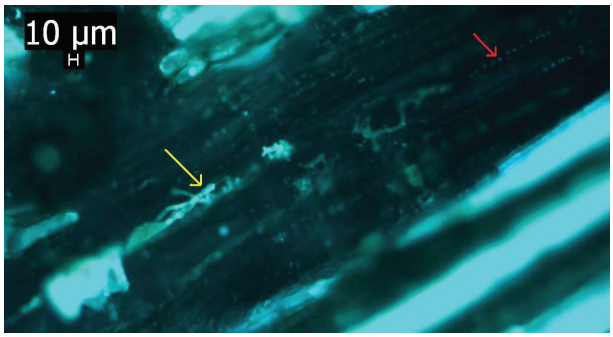

FIGURE 8: Aniline blue-stained hyphae in a sieve tube element. Fluorescence micrograph of a longitudinal section of an aniline blue-stained bark sample from a *Phytophthora ramorum* artificially infected region of a *Notholithocarpus densiflorus* log. Hyphae in inner bark in a sieve tube element (yellow arrow). Sieve areas fluoresce due to presence of callose (red arrow).

adjacent outer xylem and inner phloem were most densely colonised. Figure 9 shows hyphae in the outer xylem of a calcofluor-stained sample. Hyphal growth in the outer xylem was almost exclusively vertical. Their direction of growth in the cambium and inner bark, while primarily vertical, was also horizontal and radial. Inner bark and cambium tissues were heavily discoloured but cell walls in the xylem retained a bright blue fluorescence. Hyphae were seen in xylem vessels and xylem ray parenchyma of a calcofluor-stained sample (Figure 10) in addition to the differentiating cells in the outermost xylem.

Hyphae were seen in cork tissues with scanning electron microscopy (not shown). The small size of phellem cells and their bright autofluorescence made visualisation with calcofluor difficult.

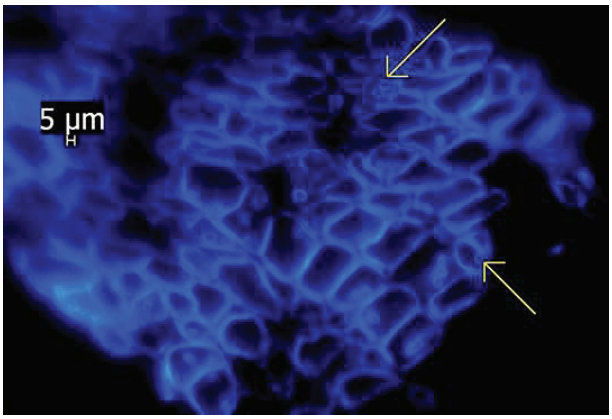

FIGURE 9: Hyphae in outer xylem. Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *Phytophthora ramorum* artificially infected region of a *Notholithocarpus densiflorus* log. Hyphae grew longitudinally, densely colonizing cells just inside the cambium in the outer xylem (arrows).

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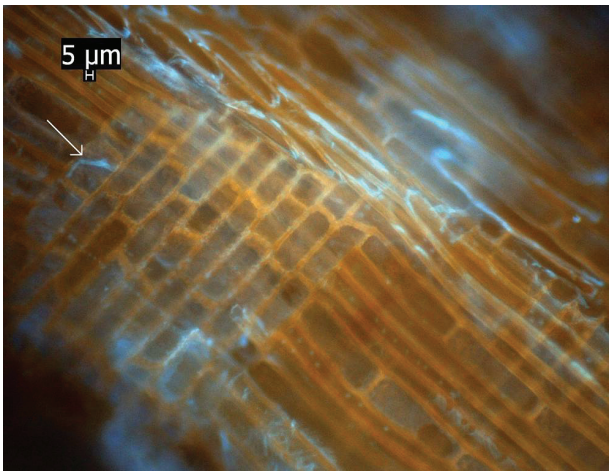

FIGURE 10: Hypha in a xylem ray (arrow). Fluorescence micrograph of a longitudinal section of a calcofluor stained *Phytophthora ramorum* infected sample from a naturally infected *Notholithocarpus densiflorus* tree.

Host responses to infection

Compared to the ordered arrangement of tissues observed in healthy control samples (Figure 11A), plant cells in infected tissues were disorganised, cells often had altered shapes, and tissues were discoloured (Figure 11B). In transverse sections, a layer of sclereids and/or fibres appeared to demarcate damaged, discoloured tissues from healthy tissues. Sclereids form the boundary in Figure 12. In artificially infected sprout samples, tissues were less severely disrupted compared to regularly shaped, turgid cells seen in healthy control tissues (Figure 13A), but cell contents were shrunken and cell shapes were distorted, as seen in Figure 13B.

Staining of callose by calcofluor occurred in all sample types including uninfected samples. Sieve areas in conducting phloem of both infected (Figure 14) and control samples were stained. In addition, small callose depositions were seen in parenchyma cells of the inner bark of both infected and uninfected tissues from artificially infected log and infected field samples (not shown). The frequency and density of these depositions, however, was much greater in infected samples. Only a few scattered, single cells in control samples showed callose. In some infected samples, most cells in infected areas were full of such depositions, both within and around infected tissue, although the frequency and density of the deposits varied. With SEM, the plasmodesmata in samples that stained positively for callose by calcofluor appeared to be plugged by a bright material (Figure 15).

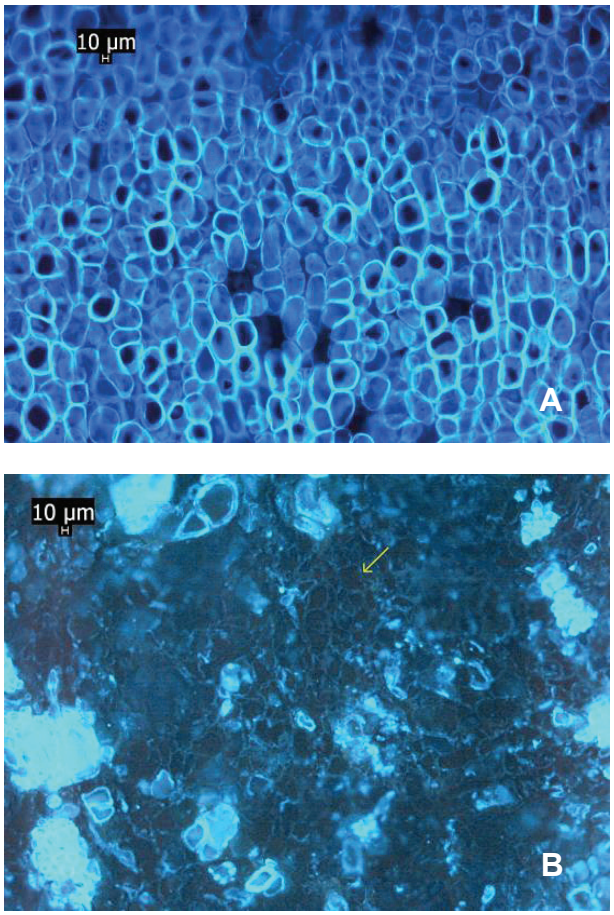

FIGURE 11: Shape and arrangement of tanoak bark parenchyma. Fluorecence micrographs of cross sections of calcofluor stained bark samples. A: Bark from healthy field-collected sample. B: *Phytophthora ramorum* infected bark parenchyma tissue. Diminished fluorescence of cell walls (yellow arrow) with calcofluor staining.

Discussion

Colonisation of *Notholithocarpus densiflorus* bark tissues

Calcofluor staining, aniline blue staining, scanning electron microscopy, and elicitin immunostaining allowed for observation of hyphal colonisation of tanoak bark tissues, demonstrating that nearly all tissues can be colonised by the pathogen. Hyphae were primarily intracellular, consistent with the *P. ramorum*/*Rhododendron* interaction as well as many other *Phytophthora* host interactions (Pogoda & Werres, 2004; Erwin et al., 1983; Keen & Yoshikawa, 1983; Klarman & Corbet, 1974). Damage to plant cells in infected tissues was evident with cell collapse, discolouration, and tissue disorganisation in artificially infected log and naturally infected field samples, and with some discolouration and plasmolysis in artificially infected sprout samples. Discolouration, cell collapse, and tissue disorganisation were also reported in

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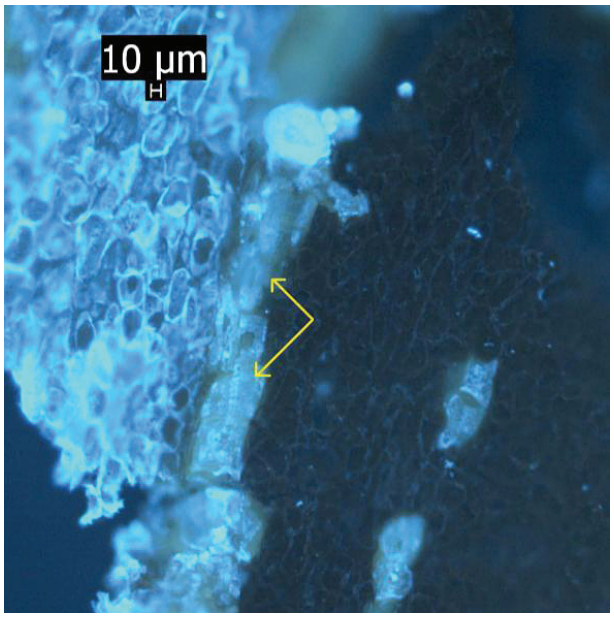

FIGURE 12: Boundary between damaged, discoloured, tissue and healthy tissue in *Phytophthora ramorum* infected bark. Fluorescence micrograph of a cross section of a calcofluor stained bark sample from a *P. ramorum* artificially infected region of a *Notholithocarpus densiflorus* log showing a boundary between healthy and damaged tissue with a layer of sclereids as the dividing line between them (yellow arrows).

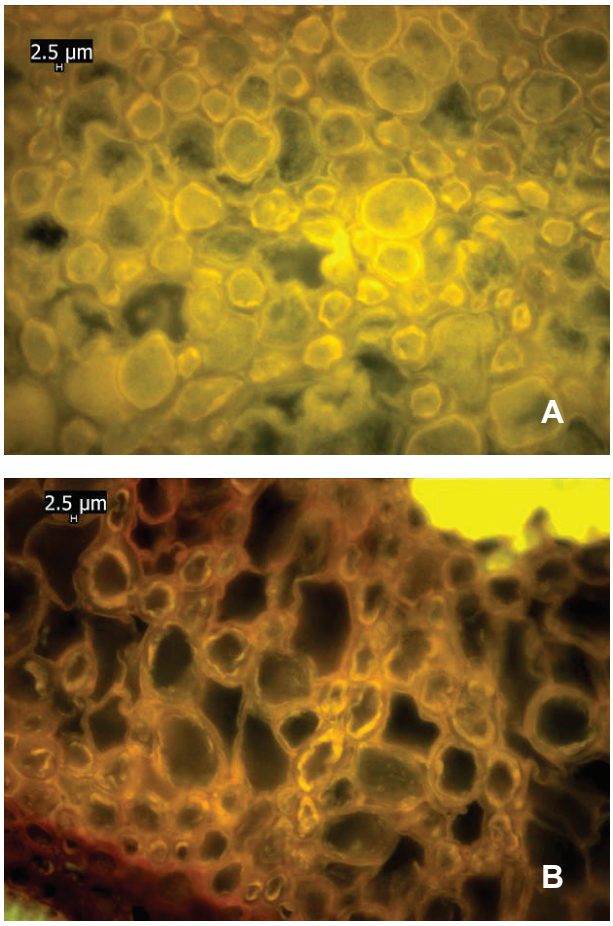

FIGURE 13: Shape and arrangement of bark parenchyma from tanoak sprout. Fluorescence micrographs of the bark of elicitin immunostained samples. A: uninfected sprout control sample. B: *Phytophthora ramorum* artificially infected sprout sample.

infected *Rhododendron* tissues, (Pogoda & Werres, 2004). Collapse of cortical cells was also reported in the interaction of *Phytophthora lateralis* and *Chamaecyparis lawsoniana* (Oh & Hansen, 2007). In susceptible *C. lawsoniana* seedlings, *P. lateralis* hyphae were reported to grow inter- and intra-cellularly in sieve cells and parenchyma cells of the secondary phloem (Oh & Hansen, 2007). This was markedly different from what was seen in resistant seedlings, where hyphae did not enter the vascular tissues (Oh & Hansen, 2007).

In *P. ramorum*-infected tissues, hyphae were seen most frequently in the phelloderm and parenchyma of the outer part of inner bark. The most obvious reason that the phelloderm may have been preferentially colonised is that many of the cells in this region in uninfected, fresh samples contained chloroplasts. Carbohydrates sequestered during photosynthesis in these tissues could serve as a food source to *P. ramorum*. Chloroplasts are seen in freshly prepared material as bright red fluorescent flecks when viewed under UV fluorescence. Chloroplasts were not visible in tissues that had been fixed and stored for a period of time.

Hyphae in the inner part of inner bark were seen in the ray parenchyma suggesting that the pathogen may move from phloem to xylem in the rays. Ray parenchyma are larger cells with thinner cell walls than most parenchyma in these tissues, and materials such as carbohydrates, minerals and nutrients are passed through these tissues. Although colonisation of xylem tissues was not investigated here, hyphae were seen in rays in outer xylem in one sample, supporting observations from *N. densiflorus* xylem (Parke et al., 2007) and *Rhododendron* phloem and xylem tissues (Pogoda & Werres, 2004) suggesting that ray parenchyma likely serve as a conduit across the cambium.

Host responses to infection

Callose in sieve areas of sieve tube elements was successfully stained in both uninfected and infected tissues by calcofluor. The presence of callose in sieve areas is well documented (Esau, 1969; Fink, 1999). Small deposits of a material that stained with calcofluor were also seen in parenchyma cells of the inner bark in both uninfected and infected samples.

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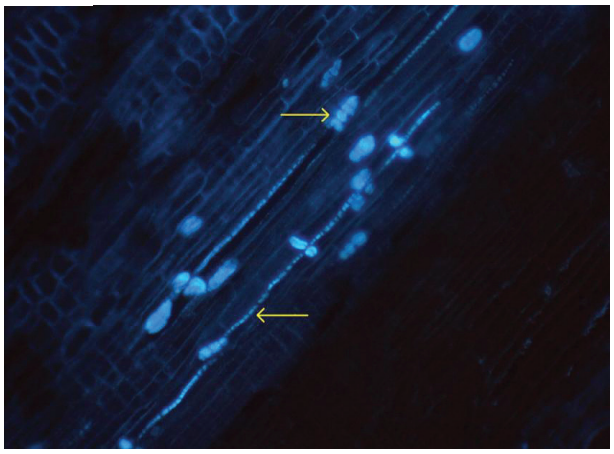

FIGURE 14: Callose in sieve tube elements (longitudinal section). Fluorescence micrograph of a longitudinal section of a calcofluor stained bark sample from a *Phytophthora ramorum* naturally infected *Notholithocarpus densiflorus* tree. Sieve areas of sieve elements fluoresce brightly due to the presence of callose (sieve areas in a sieve plate - top arrow; sieve areas on longitudinal walls - bottom arrow).

However, the frequency and density of such deposits was much greater in infected samples, though it also varied among infected samples. We suggest that these deposits represent a callose-defence response. Callose is known to be deposited in response to both wounding and pathogen infection and may be deposited specifically in pits or plasmodesmata or may be deposited more uniformly around the inside of the cell wall (Fink, 1999). We believe it is likely that callose was deposited in plasmodesmata around the walls of these parenchyma cells in infected samples, because small depositions of the material were seen.

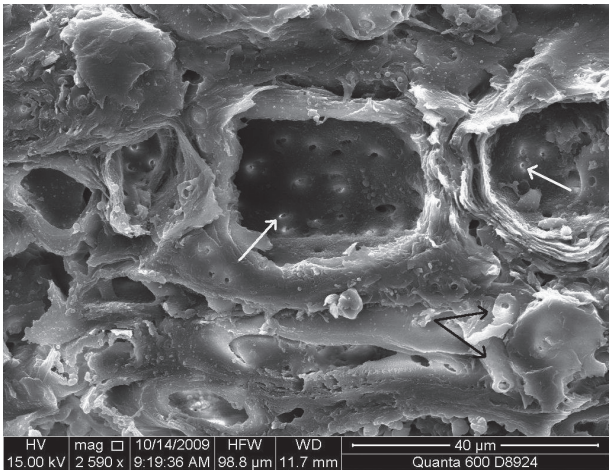

FIGURE 15: Thickenings of plasmodesmata in bark parenchyma. Scanning electron micrograph of a cross section of a bark sample collected from a *Phytophthora ramorum* infected *Notholithocarpus densiflorus* tree. Some plasmodesmata of parenchyma cells in the inner bark appear to be plugged (white arrows). There may be two hyphae below these cells (black arrows).

Apparent changes in ultrastructure, including membrane disruption and details of callose deposition could be resolved with transmission electron microscopy.

Elicitin production

Elicitin immunofluorecent labelling stained *P. ramorum* hyphal cell walls in artificially infected sprout samples. This result suggests that elicitins are localised in cell walls of *P. ramorum*, which is consistent with findings of Meijer et al. (2006). These authors showed that at least one *P. ramorum* elicitin is associated with the cell wall. In our work, in those cases where elicitin immunostaining worked, it was most visible in hyphal tips. This portion of the mycelium is where growth is most active, and elicitins are most likely produced. Although these findings do not establish a role for these proteins in pathogenicity, they would make sense within that context. If elicitins were secreted from hyphae to act as a toxin in plant cells or play some other role contributing to pathogenicity, then we would expect to find them at the hyphal cell wall and at the growing tips of hyphae or else outside of the pathogen, moving systemically through plant tissues. Elicitins were, in fact, produced in cell walls of hyphae of *P. quercina* in infected *Quercus robur* roots (Brummer et al., 2002). Manter et al., (2007) suspected that systemic movement of elicitins contributed to early declines in photosynthesis. We were unable to determine whether or not any fluorescence outside of hyphae was due to secretion of elicitins since fluorescence was also seen in controls. Further progress in this area will require more reliable immunostaining techniques.

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